Identification of Protein Interactors of Dribble — a Single KH-Domain Nucleolar Protein in *Drosophila*

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A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Philosophy
in
Molecular Biotechnology

September 2007

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DECLARATION

I declare that this dissertation is entirely my own work except where otherwise stated, either in the form of citation of published work, or acknowledgement of the source of any unpublished material.

Signed ……..

Choi Ching-Gee

ABSTRACT

In ribosome biogenesis, ribosomal ribonucleic acid (rRNA) processing proceeds with the addition of ribosomal proteins onto maturing rRNAs to form pre-ribosomal ribonucleoprotein (pre-rRNP) complexes inside the nucleolus. The *Drosophila* Dribble (DBE) protein is an evolutionarily conserved nucleolar protein which is involved in rRNA processing. Since only a K homology (KH) RNA-binding domain but no other functional domain is predicted in DBE, the molecular role of DBE is speculated to recruit other cellular proteins onto precursors of rRNA (pre-rRNA) for carrying out its role in rRNA processing. This study focuses on identifying protein interactors of DBE by an affinity pull-down approach coupled with mass spectrometric analysis. A total of 13 proteins were found to interact with DBE specifically; and 7 of them are of ribosomal proteins which have their human homologs identified in the nucleolus. Direct interaction between DBE and one of the identified ribosomal proteins, RpS9, was confirmed using an *in vitro* glutathione S-transferase (GST) pull-down assay. Meanwhile, sedimentation behavior of DBE in sucrose gradient suggests that DBE associates with a macromolecular complex *in vivo*. These findings show that DBE associates with a number of proteins including various ribosomal proteins. Further investigation on the composition of the DBE-containing macromolecular complex and the importance of DBE-ribosomal

protein interactions would better define the molecular role of DBE.

ABSTRACT (CHINESE VERSION)

在核糖體合成 (ribosome biogenesis) 的過程中，核糖體核糖核酸 (ribosomal ribonucleic acid) 於細胞核仁內進行加工，同時，核糖體蛋白 (ribosomal proteins) 與成熟中的核糖體核糖核酸結合，並形成前核糖體的核糖核蛋白複合體 (pre-ribosomal ribonucleoprotein complex)。 果蠅體內的 Dribble (DBE) 蛋白是一個進化上保守的核仁蛋白，並參與對核糖體核糖核酸的加工過程。 由於 DBE 只有一個名爲 K homology (KH) 的核糖核酸結合域 (RNA-binding domain) 而無其他預測功能域，DBE 可能帶引其他細胞蛋白到前核糖體的核糖核酸 (pre-ribosomal ribonucleic acid) 進行核糖體核糖核酸加工。 這項研究以 affinity pull-down 與質譜儀分析 (mass spectrometric analysis) 找出與 DBE 有相互作用 (interaction) 的蛋白。 結果發現共有十三個蛋白與 DBE 有相互作用；其中有七個是核糖體蛋白，而其人類同族體亦被發現於細胞核仁內。 穀胱甘肽 S-轉移酶 (glutathione S-transferase) pull-down 實驗進一步證明 DBE 和其中一個核糖體蛋白 RpS9 有直接相互作用。 此外，DBE 於蔗糖密度梯度離心法中的沉澱行爲，顯示 DBE 於細胞內存在於高分子複合體中。 綜合這項研究所得，DBE 與若干核糖體蛋白有相互作用。 進一步找出含有 DBE 的高分子複合體的組合成分，及找出 DBE 與核糖體蛋白之間相互作用的重要性，將更清晰地界定 DBE 的分子作用。

ACKNOWLEDGEMENTS

I would like extend my deepest gratitude to my supervisor Dr. Edwin H. Y. Chan for his support and patient guidance in completing this project and writing up the thesis.

I am grateful to Dr. Benny Yiu, Ms. Johanna Jim, Ms. Stephanie Ye, Ms. Winnie Pang and Mr. King-Fung Lau for their efforts in the Dribble project.

I sincerely thank all members of the Laboratory of *Drosophila* Research, CUHK, especially Ms. Priscilla Chan, Ms. Katy Chau, Dr. Macy Huen, Ms. Angel Lee, Ms. Azaria Wong, Mr. Eric Chan, Mr. Steve Lo, Mr. Alan Wong and other labmates at MMW509 and MMW507, in particular Ms. Lo-Ming Choi, Ms. Ivy Geng, Ms. Sonia Lam, Ms. Irene Lau, Ms. Peggy Law, Ms. Kit-Wan Ma, Ms. Teresa Tsang, Mr. Chi-Ho Chan, Mr. Kwok-Ho Chan, Mr. Ka-Ming Lee and Mr. Ming-Yin Lui for their help, inspiring suggestions and encouragement in all the time of my research.

I am grateful to Dr. Macy Huen, Mrs. Yeung and Mr. Denis Ip for their technical assistance on mass spectrometry.

Particularly, I would like to give special thanks to my family and friends especially my father, my mother and Mr. Alan Wong for their continuous care and support during my study.

LIST OF ABBREVIATIONS

Abbreviation used	Term
a.a.	Amino acid
APS	Ammonium persulfate
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CIAP	Calf intestinal phosphatase
CIEX	Cation exchange chromatrography
DBE	Dribble
***dbe*^{d29}**	*dribble* null mutant
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescent
EDTA	Ethylenediamine tetraacetic acid
EF1	Elongation factor 1
EGTA	Ethylene glycol-O,O'-bis (2-aminoethyl)-N,N,N',N',-tetraacetic acid
Elu	Eluent
ETS	External transcribed spacer
FLAG-DBE	FLAG-tagged Dribble
FT	Flowthrough
GF	Gel filtration
gmr	*glass multiple reporter*
GST	Glutathione-S-transferase
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-Ethanesulfonic Acid]
hnRNP	Human heterogeneous nuclear ribonucleoprotein
HRB2	HIV-1 rev-binding protein
IAA	Iodoacetamide
IPTG	Isopropyl β-D-thiogalactopyranoside
ITS	Internal transcribed spacer

KCl	Potassium chloride
kDa	Kilodalton
KH domain	K homology domain
LB	Luria-Bertani
LBA	Luria-Bertani agar
LSU	Large subunit
MALDI	Matrix-assisted laser desorption/ionization
$MgCl_2$	Magnesium chloride
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaCl	Sodium chloride
$NaCNBH_3$	Sodium cyanoborohyride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NOPdb	Nucleolar Proteome Database
NP-40	Nonidet P-40
PB	Phosphate buffer
PBS	Phosphate-Buffered Saline
PMSF	Phenylmethysulfonyl fluride
Pre-rRNA	Ribosomal ribonucleic acid precursor
Pre-rRNP	Ribosomal ribonucleoprotein precursor
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S2 cells	Schneider 2 cells
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacylamide gel electrophoresis
SSU	Small subunit
snoRNA	Small nucleolar RNA
snoRNP	Small nucleolar RNA-containing ribonucleoprotein particle
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween-20
TCA	Trichloroacetic acid

TEMED	N,N,N',N'-Di-(dimethylamino)ethane
TFA	Trifluoroacetic acid
Tris-HCl	Tris-hydrochloric acid
UAS	Upstream activator sequence
W1	First washing
W2	Second washing
WT	Wild-type

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

2. MATERIALS AND METHODS

1. INTRODUCTION

1.1 The nucleolus — a major site for ribosome biogenesis

Nucleolus is a distinctive and non-membrane-bound subnuclear compartment which has a pivotal role in ribosome biogenesis (Raska et al., 2006). It is a dynamic structure which disintegrates in early mitosis and reforms during late telophase at the genomic regions where tandemly repeated genes of ribosomal RNA (rRNA) — rDNA are located (Prieto and McStay, 2005). In the nucleolus, rRNA precursors (pre-rRNA) are actively transcribed, processed and assembled with ribosomal proteins to form large and small ribosomal subunits (reviewed by Shaw and Jordan, 1995). More than one-third of proteins identified in the human nucleolar proteome are predicted to be involved in ribosome biogenesis (Andersen et al., 2005) (Figure 1.1) and the interaction networks between these proteins are fundamental to synthesizing ribosome.

1.1.1 Ribosome biogenesis

Ribosome biogenesis involves the synthesis, maturation and sequential assembly of rRNAs with about 80 ribosomal proteins to give a large ribosomal

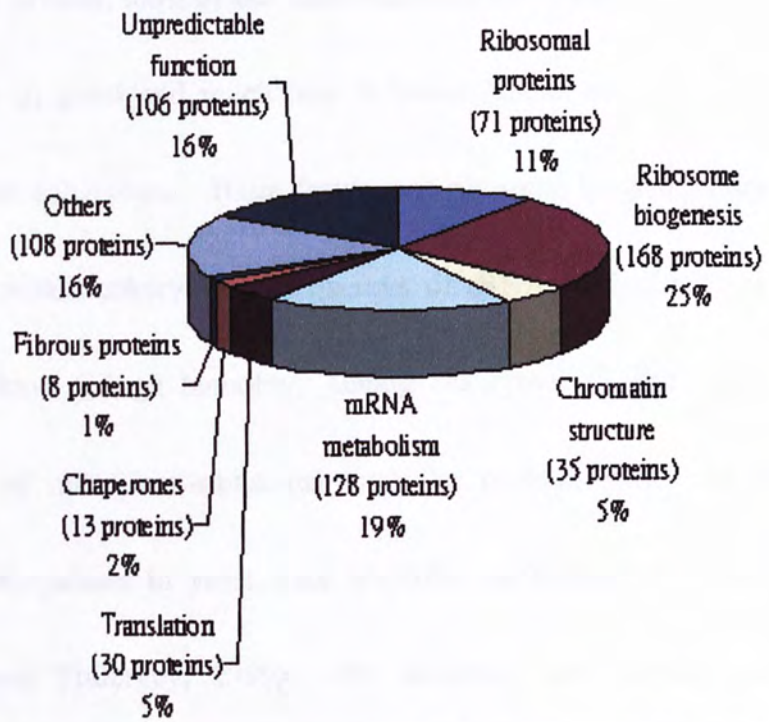


Figure 1.1 Functional classification of proteins in the nucleolar proteome. A total of 667 nucleolar proteins identified in the human nucleolar proteome (Andersen et al., 2005) were classified according to their known or predicted functions. The biological function, the percentage of proteins in the proteome and the total number of proteins for each group are given. Ribosomal proteins and non-ribosomal proteins which are responsible for ribosome biogenesis constitute more than one-third of the nucleolar proteome. (Modified from Coute et al. (2006). *Mass Spectrom Rev*, **25**, 215-234.)

subunit (60S) containing the 28S (human) /25S (yeast), 5.8S and 5S rRNAs, and a small ribosomal subunit (40S) containing the 18S rRNA (reviewed by Coute et al., 2006). At present, most of our understandings on ribosome biogenesis come from the studies in yeast and much less is known about the same process in higher multicellular eukaryotes. Basic features of ribosome biogenesis are thought to be conserved within eukaryotes. Sequences of rRNAs and ribosomal proteins were shown to have a high homology among eukaryotes (Raska et al., 2006), while homologs of many non-ribosomal nucleolar proteins which are responsible for ribosome biogenesis in yeast were identified in higher eukaryotes (reviewed by Venema and Tollervey, 1999). For example, both human nucleolar protein Fibrillarin and its yeast homolog Nop1p have a conserved role as a methyltransferase for rRNA processing (reviewed by Omer et al., 2002; Takahashi et al., 2003). In addition, the modification and cleavage patterns of pre-rRNAs were shown to be mostly conserved in eukaryotes (reviewed by Venema and Tollervey, 1999). However, distinct differences of ribosome biogenesis are present among different eukaryotes (Weinstein and Steitz, 1999). For example, the nucleolus in yeast does not disassemble during mitosis which is in contrast to what occurs in higher eukaryotes (reviewed by Raska et al., 2006). Factors which are involved in the early steps of RNA polymerase I activation are also different between yeast and

higher eukaryotes (reviewed by Raska et al., 2006). Further, the mechanism of ribosome biogenesis such as the base-pairing between U3 snoRNA and pre-rRNAs for the synthesis of 18S rRNA in multicellular eukaryotes is different from that in yeast (Borovjagin and Gerbi, 2004). Moreover, it has also been reported that the number of rRNA modifications increases with the complexity of the organism (Maden and Hughes, 1997). Therefore, the general mechanism of ribosome biogenesis found in yeast applies to most eukaryotes though it is not always possible to extrapolate the findings of ribosome biogenesis in yeast to higher eukaryotes (reviewed by Takahashi et al., 2003).

In the following parts, the two major underlying processes of ribosome biogenesis — rRNA processing (Section 1.1.1.1) and ribosome assembly (Section 1.1.1.2), and their coordinations (Section 1.1.3) were discussed.

1.1.1.1 Eukaryotic ribosomal RNA processing

Ribosomal RNA processing starts with the co-transcription of 28S/25S, 5.8S and 18S rRNAs by RNA polymerase I into a single polycistronic precursor rRNA (pre-rRNA) to give an earliest detectable 47S (human)/ 35S (yeast) pre-rRNA, and the transcription of 5S rRNA by RNA polymerase III (reviewed by Granneman and

Baserga, 2004). Immediately after transcription, pre-rRNAs undergo a series of complex post-transcriptional modifications including pseudouridylations, ribose- and base-methylations at specific sites by pseudouridine synthases and methylases (reviewed by Granneman and Baserga, 2004). Most of the modification sites on pre-rRNAs are concentrated in the conserved regions of mature rRNAs among the eukaryotes (Maden and Hughes, 1997). To cleave the 47S/35S pre-rRNA into 28S/25S, 5.8S and 18S rRNAs, two internal transcribed spacer sequences (ITS1 and ITS2) and two external transcribed spacer sequences (5′ ETS and a 3′ ETS) in the 47S/35S pre-rRNA are removed by successive exo- and endonucleolytic cleavages (Figure 1.2). Both the modifications and cleavages of pre-rRNAs are guided by a large number of snoRNAs which provides sequence specific-binding of modifying enzymes on rRNAs (reviewed by Kressler et al., 1999).

1.1.1.2 Ribosome assembly

To synthesize ribosomes, ribosomal proteins associate with maturing rRNAs in a sequential order (Fromont-Racine et al., 2003). This process is known as ribosome assembly and it begins with transcription of the nascent pre-rRNA (Dragon et al., 2002). With the continual assembly of ribosomal proteins into maturing rRNAs, it gives rise to a series of RNA-containing protein complexes which are

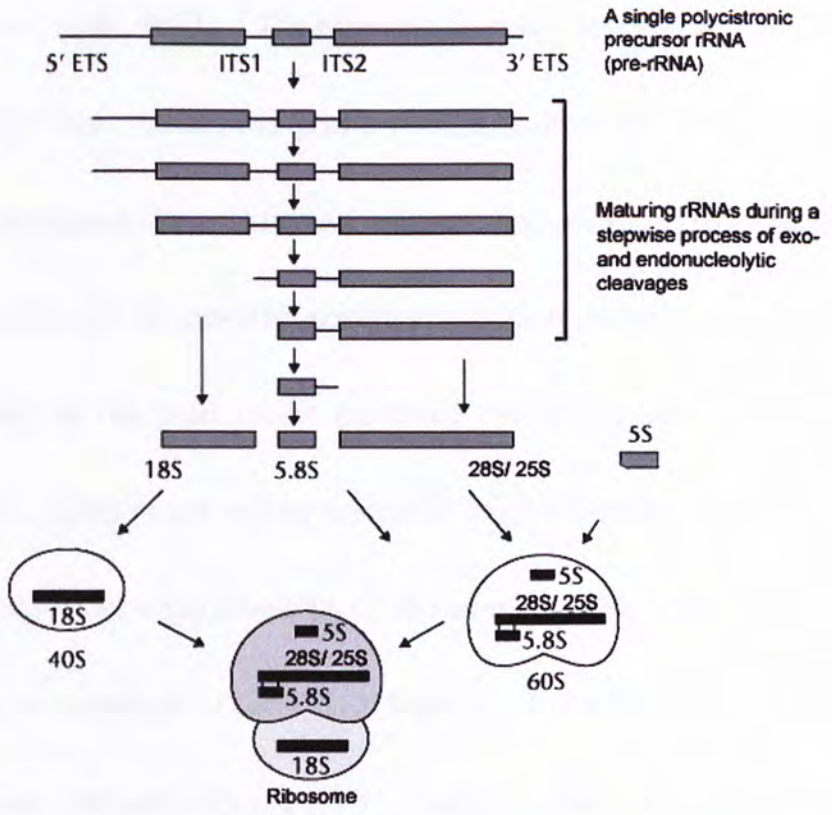


Figure 1.2 Pre-rRNA processing in eukaryotes. The structure of precursor rRNA (pre-rRNA) and rRNA intermediates are illustrated with grey boxes representing regions corresponding to mature rRNA species, and black lines representing the external transcribed spacer sequences (ETSs) and internal transcribed spacer (ITSs) sequences. Ribosomal RNA processing starts with the co-transcription of 28S/25S, 5.8S and 18S rRNAs into a single polycistronic pre-rRNA. It undergoes successive exo- and endonucleolytic cleavages at specific sites to remove ITSs and ETSs to give a mature form of 28S/25S, 5.8S and 18S rRNAs. They are assembled with other ribosomal proteins to give a large ribosomal subunit (60S) containing the 28S/25S, 5.8S and 5S rRNAs, and a small ribosomal subunit (40S) containing the 18S rRNA. (Modified from Olson (2006). *Encyclopedia of Life Sciences*. (http://www.mrw.interscience.wiley.com/emrw/9780470015902/els/article/a0005975/current/html))

known as ribosomal ribonucleoprotein precursor (pre-rRNP) complexes (reviewed by Takahashi et al., 2003). The ribosome assembly pathway was studied based on the analyses from the sucrose gradient sedimentation experiments since different pre-rRNP complexes have distinctive sedimentation properties (Schafer et al., 2003). The 90S, 66S and 43S pre-rRNP complexes are three major complexes identified in the pathway in the yeast model (reviewed by Schafer et al., 2003). The 90S pre-rRNP complex is the earliest detectable pre-rRNP complex which contains the 47S (human)/ 35S (yeast) pre-rRNA (Trapman et al., 1975; Udem and Warner, 1972). Following the cleavages of the 47S/ 35S pre-rRNA, the 90S pre-rRNP complex will split into the 66S and 43S pre-rRNP complexes which are the precursors of the mature 60S and 40S ribosomal subunits respectively (Trapman et al., 1975; Udem and Warner, 1972).

The recent success in the isolation and the proteomic analyses of a number of pre-rRNP complexes by affinity-purification and mass-spectrometry (MS) techniques reveals that ribosome assembly is a far more complicated process than the simple addition of ribosomal proteins onto pre-rRNAs (Bassler et al., 2001; Dragon et al., 2002; Grandi et al., 2002; Harnpicharnchai et al., 2001). Apart from the presence of pre-rRNAs and ribosomal proteins, there are also non-ribosomal proteins and small

nucleolar RNAs (snoRNAs) which associate with the pre-rRNP complexes. The non-ribosomal proteins and snoRNAs only associate transiently with the pre-rRNP complexes for performing their functions in ribosome biogenesis and do not remain in the mature ribosomal subunits (reviewed by Fromont-Racine et al., 2003; Takahashi et al., 2003), thus they are also described as *trans*-acting factors (Section 1.1.2). Despite the protein composition of several pre-rRNP complexes has been uncovered, the order and mechanism of the assembly of ribosomal proteins and pre-rRNAs into the pre-rRNP complexes still remain obscure (reviewed by Fromont-Racine et al., 2003; Schafer et al., 2003).

1.1.2 *Trans*-acting proteins in ribosome biogenesis

In yeast, about 170 non-ribosomal proteins and several classes of snoRNAs have been found to associate transiently with pre-rRNP complexes and they are also known as *trans*-acting factors (Coute et al., 2006; Granneman and Baserga, 2005; Nazar, 2004). Although the roles of many *trans*-acting proteins are not well characterized, their functions are generally classified into five types (reviewed by Kressler et al., 1999). The first type acts as RNA chaperones or RNA helicases to assist rRNA folding and rearrange protein-RNA interactions during rRNA processing and ribosome assembly. The second type possesses enzymatic activities or roles in

assisting enzymes for rRNA modifications (e.g. pseudouridine synthases and methyltransferases) and cleavages (e.g. endo- and exonucleases). The third type interacts with snoRNAs to give snoRNPs which function to guide processing enzymes onto the precise positions along pre-rRNAs for modifications or cleavages by base-pairing snoRNAs with pre-rRNAs. The fourth type acts as ribosome assembly factors which aid the assembly of ribosomal proteins with pre-rRNAs to give pre-rRNP complexes. The fifth type is proposed for regulating ribosome biogenesis to proceed in a well-maintained order. One example is a yeast protein named as Ssf1p, the absence of which causes premature cleavage of a specific site on pre-rRNA which should be processed later (Fatica et al., 2002). It is suggested that the association of Ssf1p with a pre-rRNP complex blocks the pre-mature cleavage of pre-rRNA whereas its disassociation from a pre-rRNP complex allows the appropriate cleavage of pre-rRNA to proceed (Fatica et al., 2002).

1.1.3 rRNA processing and ribosome assembly are two coordinated process

It is generally believed that rRNA processing and ribosome assembly are two coupled and co-regulated processes (reviewed by Granneman and Baserga, 2005; Tschochner and Hurt, 2003). Ribosomal RNA processing does not take place on "naked" rRNA transcripts, it proceeds with the addition of ribosomal proteins onto

pre-rRNAs into large pre-rRNP complexes (reviewed by Kressler et al., 1999). The formation of pre-rRNP complexes is required for efficient rRNA processing (Hitchen et al., 1997; Intine et al., 1999) and pre-rRNA cleavages begin only when the pre-rRNP complexes have formed (Kumar and Warner, 1972). In recent years, a large pre-rRNP complex termed small-subunit (SSU) processome was identified in yeast (Dragon et al., 2002). SSU processome was shown to carry an U3 snoRNA-containing ribonucleoprotein particle (snoRNP) which is responsible for initial cleavages of pre-rRNA, a subset of early-assembling ribosomal proteins and some non-ribosomal proteins (reviewed by Tschochner and Hurt, 2003). It was suggested to be the first machinery to initiate the processing of the 35S pre-rRNA, and trigger the assembly of the small ribosomal subunit (Dragon et al., 2002; Grandi et al., 2002). Thus, the presence of SSU processome suggests that rRNA processing and ribosome assembly are taken place within the same complex (Granneman and Baserga, 2005).

The question of how rRNA processing integrates with ribosome assembly and the importance of their interconnection are interesting topics for research. Studies in the yeast model showed that mutations in a large number of non-ribosomal proteins, which appears unlikely to be modifying enzymes or their co-factors, were

shown to inhibit rRNA processing (reviewed by Venema and Tollervey, 1999). Some of these proteins were proposed to associate with the assembly of pre-rRNP complexes (Jin et al., 2002). One specific example is nucleolin. It is a non-ribosomal protein which is involved in the first cleavage step of rRNA processing to remove the 5' ETS (Ginisty et al., 2001) but it has no nuclease activity (Roger et al., 2002). The involvement of nucleolin in rRNA processing was suggested to be related to its ability to assemble ribosomal proteins and pre-rRNA processing factors into pre-rRNP complexes (Allain et al., 2000; Bouvet et al., 2001; Bouvet et al., 1998; Liu and Yung, 1999). Increase amount of nucleolin was shown to cause incorrect assembly of 40S ribosomal subunit and defects in rRNA processing (Roger et al., 2003). This finding showed that ribosome assembly and rRNA processing are two interdependent processes.

1.2 Dribble (DBE) — an essential nucleolar RNA-binding protein

Dribble (DBE) is a *Drosophila* nucleolar protein which is encoded by an essential gene called *dbe* (reviewed by Chan et al., 2001). Mutations in *dbe* are lethal; homozygous *dbe* null mutants die at the first-instar larval stage of the *Drosophila* life cycle. The larval lethality was suggested to be caused by the exhaustion of maternal gene product of *dbe*. Expression of *dbe* is ubiquitous in

embryonic cells, and it appears to be required for cell viability during development. The DBE protein is preferentially localized to the perinucleolar ring structure of the nucleus. It is a 345-amino acid-long protein which carries a single K homology (KH)-domain (Section 1.2.1) located at the 136^{th} to 188^{th} amino acid residues of its sequence. DBE is an evolutionarily conserved protein and 22 homologs of DBE have been identified (Xin et al., 2005). They include HIV-1 Rev-binding protein (HRB2) in human, Mis3 in fission yeast (Kondoh et al., 2000), and Krr1p in budding yeast (Xin et al., 2005). Among them, DBE has been the most characterized homolog in multicellular eukaryotes.

1.2.1 DBE carries a RNA-binding KH-domain

DBE protein carries a KH-domain (Chan et al., 2001) which is an evolutionarily conserved RNA-binding motif that is present in eubacteria and eukaryotes (reviewed by Messias and Sattler, 2004). It was first identified in the human heterogeneous nuclear ribonucleoprotein (hnRNP) K (Siomi et al., 1993). It was later found in a wide range of nucleic acid-binding proteins and was recognized as one of the most common single-stranded RNA-binding domains after the RNA recognition motif (RRM) (reviewed by Chen and Varani, 2005). The KH-domain is of approximately 70-amino acid-long, and the most conserved region lies in the middle of the domain

and with the sequence of (I/L/V)-I-G-X-X-G-X-X-(I/L/V) (Siomi et al., 1993). The structure of KH-domain consists of an αβ fold (Ramos et al., 2002). The nucleic-acid recognition surface of the KH-domain is located at the narrow groove formed between the consecutive α-helices which are connected by a highly conserved Gly-X-X-Gly loop (reviewed by Auweter et al., 2006; Grishin, 2001). A single amino acid mutation of the first glycine in this consensus sequence of a KH-domain-containing protein Sam68 impairs its RNA-binding property (Chen et al., 1997). KH-domain can occur singly or in multiple copies in a protein (Chen et al., 1997; Ramos et al., 2002). DBE and all its homologs are found to carry a single copy of KH-domain (Chan et al., 2001; Gromadka and Rytka, 2000; Xin et al., 2005).

KH-domain was found in proteins of diverse functions (reviewed by Grishin, 2001). For example, ribosomal protein S3 (Siomi et al., 1993) and Dim2p are involved in ribosome biogenesis (Vanrobays et al., 2004); whereas hnRNP K is involved in the regulation of gene expression (Siomi et al., 1993).

1.2.2 RNA-binding properties of DBE

The RNA-binding ability of purified DBE protein was studied *in vitro* using

RNA homo-polymer resins (Yiu et al., 2006). DBE was shown to interact with poly-U, poly-A, and poly-C RNA homo-polymers at similar affinity; and the binding between DBE and RNA homo-polymers was shown to be inhibited by an RNA analogue heparin (Yiu et al., 2006). It was speculated that the KH-domain in DBE is responsible for RNA-binding (Chan et al., 2001; Yiu et al., 2006). However, apart from the KH-domain, DBE might also associate with RNA through its disorder structure (Yiu et al., 2006). Bioinformatic analysis revealed that 46% of the DBE sequence is disordered (Yiu et al., 2006). Disorder structure has been implicated in RNA-binding in other proteins (Fink, 2005; Tompa, 2002). As predicted, DBE displays a proteolytic sensitive characteristic (Yiu et al., 2006) that is a common feature of a disorder protein (Tompa, 2002). The RNA-binding ability of the disorder sequence is demonstrated by the protection of DBE from protease digestion upon its binding with an RNA analogue heparin as it is suggested that the disorder sequence in DBE is shielded away from protease when it binds heparin (Yiu et al., 2006).

The RNA-binding specificity of purified DBE protein was also studied *in vitro* and it showed that purified DBE protein can bind to various types of RNA including pre-rRNA, rRNA, mRNA and tRNA (Lau, Pang and Chan, unpublished data). The

non-specific affinity of DBE towards different types of RNAs had raised the question of whether additional protein factors are required by DBE to achieve specificity in RNA-binding.

1.2.3 DBE is involved in rRNA processing

The involvement of *dbe* in rRNA processing was demonstrated by Northern blot analysis over RNA extracted from a *dbe* null mutants (dbe^{D29}) which had the whole open reading frame of *dbe* deleted (Chan et al., 2001). The analysis showed *dbe* mutant larvae has aberrant pre-rRNA species, and an overall reduction of most pre-rRNA intermediates and mature rRNAs was also observed.

To illustrate the rRNA processing defects found in *dbe* mutants, an outline of rRNA processing in *Drosophila* was shown in Figure 1.3A. In the normal rRNA processing, the intermediate "a" is generated in the first cleavage step at site 1 for removing 5' ETS (Figure 1.3A). Northern blot analysis showed that *dbe* mutants have an aberrant pre-rRNA species which is slightly shorter than the intermediate "a" (Figure 1.3B). This suggested that depletion of DBE may either cause the lost in specificity of the first pre-rRNA cleavage at site 1 (Figure 1.3A) to generate intermediate "a" or it may cause further undesired cleavage along the intermediates

A

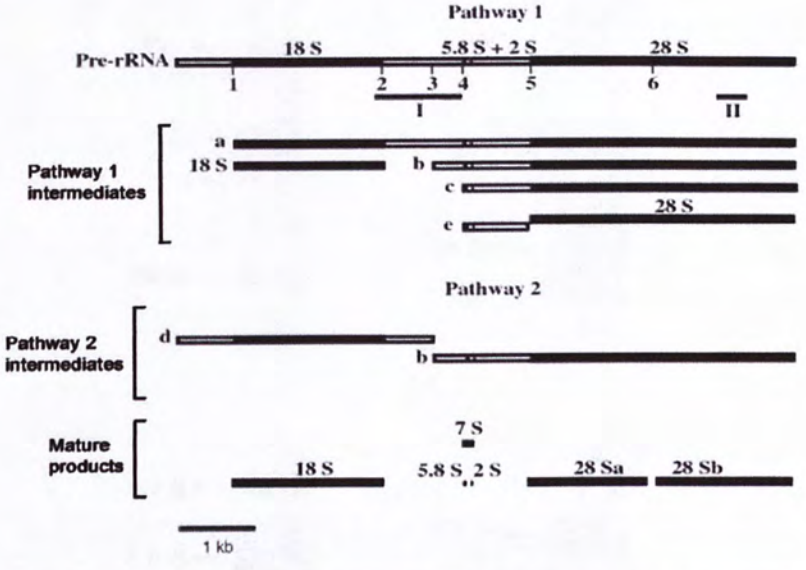


Figure 1.3 The involvement of DBE in pre-rRNA processing. **(A)** The outline of pre-rRNA processing in *Drosophila* is shown. The structure of pre-rRNAs are illustrated with solid boxes representing regions corresponding to mature rRNA species, and the open boxes representing the ETS and ITS. The structure of the pre-rRNA transcript is shown and the numbers along it denote the endonucleolytic sites. The small lines (I and II) below the pre-rRNA transcript indicate the positions of probes used in Northern Blotting analysis. Two pathways of pre-rRNA processing are present in *Drosophila*. In pathway 1, it generates intermediates "a", "b", "c" and "e". In pathway 2, it generates intermediates "b" and "d".

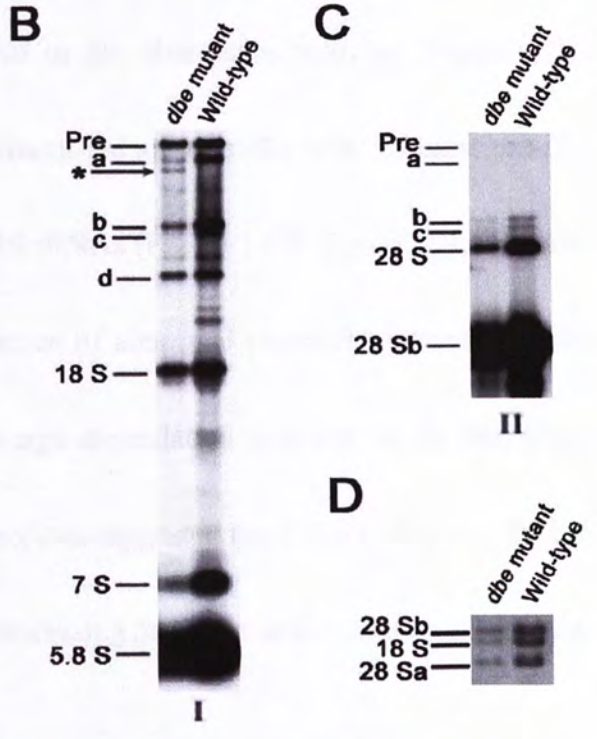


Figure 1.3 (continued) (B) The Northern blot of total nucleic acids purified from wild-type and *dbe* mutant using probes I is shown. The identities of rRNA-processing intermediates are indicated at the left of the gel photos. The mutant *dbe* had an aberrant pre-rRNA species which is slightly shorter than an early pre-rRNA intermediate denoted as "a". The aberrant intermediate is indicated with asterisk and arrow. **(C)** The Northern blot of total nucleic acids purified from wild-type and *dbe* mutant using probes II is shown. **(D)** The levels of rRNA between wild type and *dbe* mutant are shown. The overall level of mature rRNAs decreased in *dbe* mutant. (Modified from Chan et al. (2001). *Mol Biol Cell*, **12**, 1409-1419.)

"a" to give an aberrant pre-rRNA species (Chan et al., 2001). In an alternative processing pathway, "b" and "d" intermediates are generated in the initial cleavage at site 3 (Figure 1.3A). The level of "d" is not affected in *dbe* mutants suggesting that *dbe* is not involved in the alternative pathway (Figure 1.3B). In addition, *dbe* mutants have an overall reduction in the level of most pre-rRNA intermediates and mature 18S and 28S rRNAs (Figure 1.3B; Figure 1.3C; Figure 1.3D). This may be the direct consequence of abnormal pre-rRNA processing since aberrant pre-rRNA species might undergo degradation and not be further processed to give mature rRNAs. Therefore, it is suggested that DBE is required for the first cleavage step in one of the rRNA processing pathways in *Drosophila* (Chan et al., 2001).

The molecular role of DBE in the early cleavage step in rRNA processing is yet to be found. Previous study showed rRNA processing is a complicated process which requires a large number of components to carry out extensive modifications and cleavages at precise sites (Section 1.1.1.1). Since no enzymatic domain for RNA metabolism has been found in DBE so far, DBE may act as a RNA chaperone for maintaining a proper structure of rRNA for processing or it might interact with other protein factors to bind specific sequence of rRNA for directing processing factors to the precise processing sites of pre-rRNA. The RNA-binding property of

DBE suggests it might bind to pre-rRNA or snoRNA (Section 1.1.1.1). DBE might associate with snoRNA with other proteins to form snoRNP which might be the U3 snoRNP that is also involved in the removal of 5′- ETS from pre-rRNA (Sharma and Tollervey, 1999). Alternatively, like another single KH-domain protein Dim2p which recruits a dimethyltransferase Dim1p to pre-rRNA for methylations through its KH-domain (Vanrobays et al., 2004), DBE might interact with a nuclease and direct it onto pre-rRNA for cleavages. However, in the case of Dim1p and Dim2p, it showed that modification patterns of pre-rRNA can also affect the subsequent cleavage step in rRNA processing as the mutants of *dim1* and *dim2* give unmethylated 22S rRNA which results in the accumulation of aberrant rRNA intermediate (Vanrobays et al., 2004). Thus, DBE could also implicate in the modification of rRNA by recruiting modifications enzymes to pre-rRNA.

1.2.4 Yeast homolog of DBE — Krr1p is associated with ribosome biogenesis

The Yeast homolog of DBE — Krr1p is localized to the nucleolus and essential for cell viability (Gromadka et al., 1996; Sasaki et al., 2000). Interaction study of Krr1p found that it interacts functionally and physically with many ribosomal proteins (Gavin et al., 2006; Grandi et al., 2002; Gromadka et al., 2004) and it was also found to be a bona fide component of the small-subunit processome

(SSU processome) which is an early pre-rRNP complex required for the biogenesis of the 18S rRNA (Bernstein et al., 2004). Krr1p is involved in rRNA processing as depletion of Krr1p was shown to cause defect in 18S rRNA synthesis and lead to reduction of 40S ribosomal subunits (Gromadka and Rytka, 2000; Sasaki et al., 2000). In contrast to the *dbe* mutants which have depleted levels of mature 28S and 28Sb rRNA (Chan et al., 2001), mutations in *krr1* lead to the depletion of 18S rRNA only but not 25S rRNA which is equivalent to the 28S and 28Sb rRNA in *Drosophila* (Figure 1.4). Nevertheless, the pulse-chase labeling experiment showed that 25S rRNA in the *krr1* mutant was produced at a slower rate than the wild type which indicates Krr1p might still be required for the maturation of 25S rRNA (Figure 1.4). The difference of defects in the rRNA processing between *dbe* and *krr1* mutants might be due to the cross-phylum variations in rRNA processing (Chan et al., 2001). Moreover, Krr1p was reported to interact with another nucleolar protein — Faf1p, which has no clear homolog in higher eukaryotes including *Drosophila* (Karkusiewicz et al., 2004). The mutant of *faf1* has decreased levels of 18S rRNA and 40S ribosomal subunit (Karkusiewicz et al., 2004) which is similar to *krr1* mutant. It is possible that the interaction between Faf1p and Krr1p is important for performing rRNA processing in yeast. Thus, the lost of *faf1* during evolution may explain the difference of the rRNA processing defects between *dbe* and *krr1* mutants.

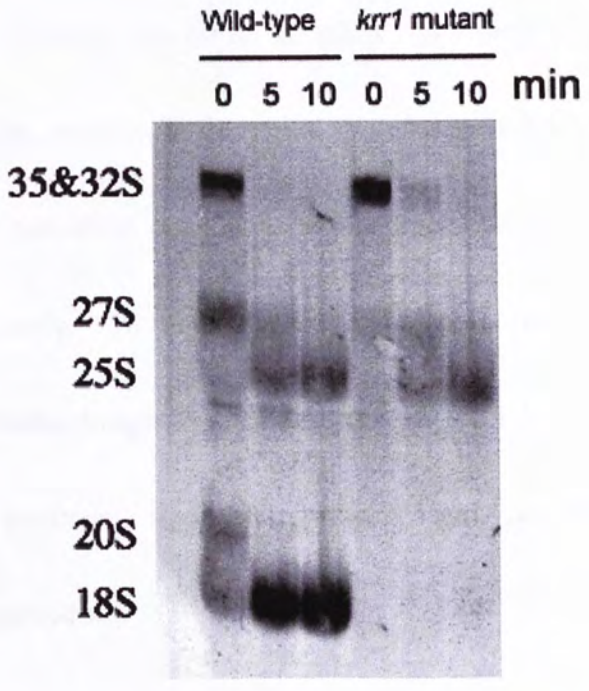


Figure 1.4 The involvement of *krr1* in pre-rRNA processing. The result of pulse-chase labeling of rRNAs using wild-type yeast and *krr1* mutant was shown. Newly synthesized rRNAs were labeled with [*methyl*-3H] methionine. At the time point of 5-min and 10-min after the pulse-chase labeling experiment started, 18S rRNA was produced in the wild-type yeast but not in *krr1* mutant. 25S rRNA was synthesized in both the wild-type and *krr1* mutant; however, 25S rRNA was produced at a slower rate in the *krr1* mutant than the wild-type. (Modified from Sasaki et al. (2000). *Mol Cell Biol*, **20**, 7971-7979.)

1.3 Aims of Research

Ribosome biogenesis involves two major and coordinated processes — rRNA processing and ribosome assembly which require a large number of non-ribosomal proteins. The precise functions of many non-ribosomal proteins are poorly understood. Also, many non-ribosomal proteins have been identified to associate transiently with pre-rRNP complexes which might be important for their roles in ribosome biogenesis. To understand the molecular functions of non-ribosomal proteins in ribosome biogenesis, finding the physical interaction networks among non-ribosomal proteins, ribosomal proteins and pre-rRNAs is one of the indispensable approaches.

DBE is one of the non-ribosomal nucleolar proteins which carries an RNA-binding domain and is involved in rRNA processing. Previous study demonstrated *dbe* mutants lose the rRNA cleavage specificity which gives aberrant pre-rRNA intermediates and depleted levels of mature rRNAs. Since DBE lacks any known enzymatic domain in its sequence to explain for its processing defects in its mutants, the RNA-binding domain in DBE suggests DBE might help to recruit enzymatic factors onto the correct processing sites along pre-rRNAs. In additions, DBE may associate with pre-rRNP complex like other non-ribosomal proteins for

ribosome biogenesis. To verify these hypotheses, this study focuses on finding the protein interactors of DBE by affinity pull-down, confirming interactions between DBE and protein interactors identified in the affinity pull-down, and investigating whether DBE exists in a complex by studying the sedimentation behavior of DBE in sucrose gradient sedimentation.

2. MATERIALS AND METHODS

2.1 Expression and purification of DBE protein in *Escherichia coli*

2.1.1 DNA construct

The construct for DBE expression is the pET-3a-*dbe* plasmid which has an open-reading frame of *dbe* subcloned into the expression vector of pET-3a (Novagen) (Yiu et al., 2006).

2.1.2 Bacterial culture

To overexpress DBE protein, the pET-3a-*dbe* construct was used to transform *Escherichia coli (E. coli)* C41(DE3) cells (Miroux and Walker, 1996). Bacterial colonies transformed with pET-3a-*dbe* were selected on Luria-Bertani broth (LB) agar plate containing 100 μg/ml ampicillin (Section 2.13.1). To obtain a starter culture, a single transformed colony was inoculated to 0.5 ml of LB medium (Section 2.13.1) containing 100 μg/ml ampicillin and 2% (w/v) glucose, and the cells were allowed to grow for approximately 4 hr at 37°C. A volume of 10 ml starter culture was then used to inoculate 1 litre of fresh LB medium containing 100 μg/ml

ampicillin. The bacterial cells were grown at 37°C at 250 revolutions per min (rpm) until the OD_{600} reading reached 0.4 to 0.6 unit. Dribble protein expression was induced by adding 250 µl of 0.4 M of Isopropyl β-D-thiogalactopyranoside (IPTG; 0.1 mM; Section 2.13.1) to 1 litre of LB broth. The bacterial cells were allowed to grow overnight at 22°C at 250 rpm. Cells were harvested by centrifugation at 3,700 X g (Beckman rotor, JLA 16.25) for 10 min at 4°C. The bacterial cell pellet was then resuspended in buffer A (40 ml per litre culture; Section 2.13.2) containing 0.5 mM Phenylmethyl Sulfonyl Floride (PMSF) and 0.1% (v/v) β-mercaptoethanol. The cell suspension was sonicated on ice for ten 30-sec cycles with a 1 min break between each cycle. To obtain a soluble protein fraction, the bacterial cell lysates were centrifuged at 14,000 X g (Beckman rotor, JA30.5) for 30 min at 4°C. The supernatant was collected and filtered through a 0.22-µm filter membrane (Millipore) prior to protein purification (Section 2.1.3).

2.1.3 Purification of DBE protein

Cation exchange chromatography was carried out to purify the overexpressed DBE protein from the soluble bacterial lysates (Section 2.1.2). A 5-ml Hi-Trap SP HP cation exchange column (Amersham Biosciences) was used, and operated through the AKTA Explorer system (Amersham Biosciences). The flow rate was

maintained at 5 ml/min, and the protein content of the eluent was monitored by OD_{280} absorbance. Prior to each run, the column was first washed with 5X column volumes of buffer B (Section 2.13.2), and then equilibrated in 5X volumes of buffer A (Section 2.13.2). The soluble bacterial lysates were then applied to the column and the column was washed with buffer A until the OD_{280} absorbance returned to the baseline level. A linear sodium chloride (NaCl) gradient of 0 to 1 M NaCl was used to elute proteins at a flow rate of 5 ml/min. The DBE protein was eluted at 50-60 mM NaCl. After the gradient elution, the column was washed with 5 column volume of buffer A and then 5 column volume of 20% (v/v) ethanol. The column was stored in 20% (v/v) ethanol. Based on the OD_{280} reading of the chart recorder, fractions referring to the peak of OD_{280} were selected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Section 2.6). Corresponding DBE-containing fractions collected from the Hi-Trap SP HP column were pooled and concentrated to approximately 10 ml using ultra-centrifugal filter device (Millipore) with a molecular cut-off value of 10 kDa. Size exclusion chromatography was employed as the second step for protein purification. A Hi-Load Superdex 200 prep grade column (Amersham Biosciences) was used. After the column was equilibrated with the elution buffer (Section 2.13.2), protein sample was loaded to the column at a flow rate of 2.5 ml/min. The DBE protein

was eluted at 180 ml elution volume after protein injection. Purified DBE protein in the elution buffer was kept as aliquots of 200 µl and stored at -80°C. Some purified DBE protein was subjected to heat-denaturation by boiling at 99°C for 15 min. Proteins were then centrifuged at 16,100 X g for 5 min at 4°C to remove any precipitated proteins.

2.2 *Drosophila* cell culture and preparation of protein lysates

2.2.1 *Drosophila* S2 cell culture

Drosophila S2 cells (Schneider, 1972) were grown at 25°C in Schneider's *Drosophila* medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen) and 100 units/ml penicillin G sodium (Hyclone) plus 100 mg/ml streptomycin sulfate (Hyclone).

2.2.2 Preparation of protein lysates from *Drosophila* cultured cells

Drosophila S2 cells grown in 25-cm^2 canted flask were harvested by cell scraper (Spl life sciences), washed twice with ice-cold 1X phosphate-buffered saline (PBS; Section 2.13.14), and then resuspended in 400 µl ice-cold hypotonic lysis buffer (Section 2.13.3). The cell suspension was sonicated on ice for six 5-sec

cycles and with a 5-sec break between each cycle. To clear cell debris, cell lysates were centrifuged at 16,100 X g for 5 min at 4°C.

2.2.3 Ribonuclease A (RNase A) treatment of protein lysates from *Drosophila* cultured cells

To remove RNA from protein lysates, 400 µl of the protein lysates obtained from *Drosophila* cultured cells were incubated with 50 µg/ml RNase A (Sigma) for 30 min at 37°C.

2.3 *Drosophila* culture and genetics

2.3.1 *Drosophila* culture

Fly stocks were maintained in plastic vials containing cornmeal-yeast-glucose-agar medium (Section 2.13.4). Fly stocks were kept at room temperature or in 25°C incubators (LMS). Distilled water was added to stocks to prevent fly food from drying out. Virgin female flies required for setting up genetic crosses for experiments were collected within 8 hr at 25°C or 16 hr at 18°C after the fly vial was cleared of adults. All crosses were raised at 25°C.

2.3.2 GAL4/UAS transgene expression in *Drosophila*

To overexpress DBE or FLAG-tagged DBE protein in *Drosophila*, the *gmr*-GAL4/UAS system was utilized to express transgenes in the *Drosophila* eye (Ellis et al., 1993 and Phelps and Brand, 1998). The *dbe* transgene was placed downstream of a UAS (upstream activating sequence) that consists of GAL4-binding sites. GAL4 is a yeast transcriptional activator. In the presence GAL4, the transgene would be expressed in *Drosophila*. *Glass multimer reporter* (*gmr*) is an eye specific promoter situated upstream of the *GAL4* gene which gives the production of GAL4 in *Drosophila* eye for activating the expression of transgene. Flies carrying both the UAS-*dbe* or UAS-FLAG-*dbe* and the *gmr*-GAL4 transgene will overexpress the transgenic DBE protein in the *Drosophila* eye. Fly heads were decapitulated to obtain protein lysates for sucrose gradient sedimentation (Section 2.10), immunoprecipitation of FLAG-DBE (Section 2.12) and calf intestinal phosphatase treatment (Section 2.13).

2.4 Affinity pull-down

2.4.1 Immobilization of proteins onto AminoLink Plus coupling beads

To generate baits for the affinity pull-down, the AminoLink Plus coupling resin

beads (Pierce) were used to immobilize purified DBE protein (Section 2.1.3), lysozyme (Sigma) and heat-denatured DBE protein (Section 2.1.3.1). The resin beads were linked directly with aldehyde groups which would react with primary amines of proteins to form reversible Schiff's bases (Figure 2.1). With the addition of sodium cyanoborohydride for the reductive amination of the double-bonds in the Schiff's bases, a permanent covalent bond would be formed between proteins and beads (Figure 2.1). In the procedures for protein immobilization, all centrifugation steps were performed at 800 X g for 3 min at room temperature. The resin beads were first washed with 300 µl of coupling buffer (Section 2.13.5) for 3 times with brief vortex mixing. Two hundred micrograms of purified DBE protein, lysozyme (Sigma) and heat denatured DBE protein which were diluted 2-fold in coupling buffer, then added respectively to 200 µl of coupling beads in a 1.5-ml microfuge tube. Two microlitre of 5 M sodium cyanoborohyride ($NaCNBH_3$, Pierce) in 0.01 M sodium hydroxide (NaOH) was added to the tube for the crosslinking between proteins and beads. The tube was incubated for 4 hr with end-over-end rotation at room temperature for coupling reaction. After the incubation, coupling beads were collected by centrifugation. The beads were then washed with 300 µl of coupling buffer for 3 times to wash away all uncoupled protein. The beads were then washed with 300 µl of quenching buffer (Section 2.13.5) twice. To block the

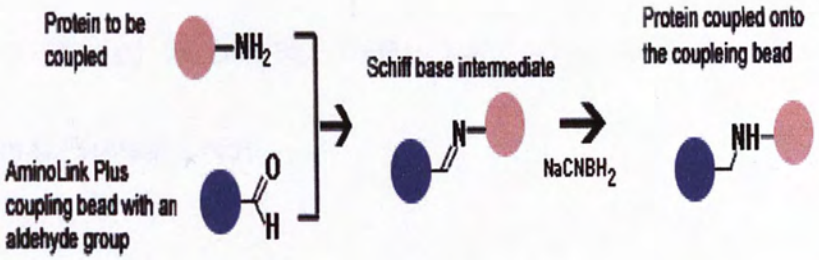


Figure 2.1 The principle of immobilization of proteins onto AminoLink Plus coupling resin beads. AminoLink Plus coupling resin beads are linked directly with aldehyde groups which react with primary amines of proteins to form reversible Schiff's bases. The addition of sodium cyanoborohydride catalyzes reductive amination of the double-bonds in the Schiff's bases and a permanent secondary amine linkage is formed between proteins and beads.

remaining active sites on the coupling beads, 200 µl of quenching buffer and 4 µl of sodium cyanoborohyride were added to the coupling beads. The tube was incubated for 30 min with end-over-end rotation at room temperature for quenching reaction. After the incubation, the beads were collected by centrifugation. The beads were washed with 300 µl of washing solution (Section 2.13.5) for 3 times and then with 300 µl of coupling buffer for 3 times prior to affinity pull-down experiments (Section 2.4.2).

2.4.2 Affinity pull-down using protein coupled-beads

A volume of 350 µl of 2-fold diluted *Drosophila* S2 cells (Section 2.3.1) was added to the DBE-coupled beads for affinity pull-down. All centrifugation steps throughout this procedure were performed at 800 X g at 3 min at room temperature. Interaction between DBE and *Drosophila* S2 protein lysates was allowed to take place in a 1.5-ml microfuge tube for 2 hr with end-over-end rotation at room temperature. After the incubation, coupling beads were collected by centrifugation. The beads were washed 6 times with 300 µl of washing buffer (Section 2.13.6). Proteins bound on the DBE coupled-beads were eluted by adding 300 µl of elution buffer (Section 2.13.6) and incubated for 5 min with end-over-end rotation at room temperature. Flow-through, washes and eluents were subjected to Trichloroacetic

acid precipitation (Section 2.5).

2.5 Trichloroacetic acid protein precipitation

Trichloroacetic acid (TCA; Sigma) was used to precipitate and concentrate proteins. Precipitation was carried out overnight in the presence of 13% (v/v) TCA at -20°C. TCA-precipitated protein samples were collected by centrifugation at 16,100 X g for 45 min at 4°C. Supernatant was discarded and the protein pellet was washed in 300 µl of ice-cold acetone with vigorous vortex mixing. The sample was then centrifuged again at 16,100 X g for 30 min at 4°C and supernatant was discarded. The washing step was performed twice. Finally, the protein pellet was then dissolved in 6X SDS sample buffer (Section 2.13.7) and stored at -20°C.

2.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gels were performed using the Mini-PROTEAN III (Bio-Rad Laboratories) and PROTEAN II xi (Bio-Rad Laboratories) electrophoresis cell. Glass plates of 0.75-mm or 1.5-mm spacers were used in Mini-PROTEAN III electrophoresis cell whereas 20-cm long and 1.0-mm thick spacers were used in PROTEAN II xi electrophoresis cell. Gel cassettes were assembled according to

manufacturer's instructions. Separating gel solution was prepared (Section 2.13.7) and loaded into the gap between the assembled glass plates. Isopropanol was loaded onto the top of the separating gel solution to remove any trapped air bubbles and to keep the gel solution out of atmospheric oxygen. The running gel solution was allowed to polymerize for 20 min at room temperature or until the gel was set. Isopropanol was removed from the running gel surface. The gel surface was rinsed with double distilled water, and dried by placing a piece of filter paper near the gel surface. The stacking gel solution was prepared (Section 2.13.7) and then loaded onto the top of the running gel. A comb of appropriate thickness with 10, 15 or 25 wells was inserted between the glass plates. Stacking gel solution was allowed to polymerize for 20 min at room temperature or until the gel was set. The gel cassette was then transferred into the electrophoresis cell. The inner cell chamber and outer cell chamber were filled with 1X SDS electrophoresis buffer (Section 2.13.7). Prior to sample loading, the comb was removed and the wells were rinsed with fresh 1X SDS electrophoresis buffer. Protein samples were denatured by boiling at 99°C for 5 min, and loaded into the wells. For each gel, 5 µl (Mini-PROTEAN III) or 10 µl (PROTEAN II xi) of prestained protein standard (Bio-Rad Laboratories) was also loaded onto the gel as molecular marker. Electrophoresis was conducted at constant voltage of 80 V (Mini-PROTEAN III) or 100 V (PROTEAN II xi) until the dye front

has passed through the stacking gel into the separating gel. Electrophoresis was continued at 120 V (Mini-PROTEAN III) or 200 V (PROTEAN II xi) until the dye front reached the desired distance which was optimal for the separation of proteins. The gel was then removed from the gel cassette of the electrophoresis cell and rinsed with double distilled water.

2.7 Coomassie blue staining and destaining

The SDS-PAGE gel was stained by immersing the gel in Coomassie blue staining solution (Section 2.13.8) and incubated overnight at room temperature with gentle shaking. To destain the SDS-PAGE gel, the Coomassie blue staining solution was removed and replaced with destaining solution (Section 2.13.8). The gel was destained at room temperature with gentle shaking. The destaining solution was discarded when it appeared dark blue. Destaining was continued by replacing fresh destaining solution until the background staining of the gel disappeared.

2.8 Protein identification by tandem mass spectrometry

2.8.1 In-gel trypsin digestion of protein bands

Protein bands of interest were excised from the Coomassie blue-stained

SDS-PAGE gel by 25G needles (Terumo). Each gel band was further cut into smaller pieces of about 1-mm cubic size and put in a 1.5-ml microfuge tube, was then destained with 200 µl of destaining solution overnight at room temperature. Destaining solution was discarded and replaced with fresh destaining solution to further destain the gel pieces and destaining was repeated until the gel pieces appeared colorless. A volume of 200 µl of acetonitrile (Sigma) was added to dehydrate the gel pieces for 10 min until the gel pieces became opaque-white in color. The acetonitrile was then removed by vacuum centrifugation (Speed Vac System, Savant) for 5 min at room temperature. A volume of 50 µl of freshly prepared 10 mM dithiothreitol (DTT; Section 2.13.9) in 100 mM ammonium bicarbonate (Section 2.13.9) was added to perform in-gel reduction for 30 min at room temperature. Then, the DTT was discarded and 50 µl of 100 mM Iodoacetamide (IAA; Section 2.13.9) in 100 mM ammonium bicarbonate was added for in-gel alkylation for 30 min at room temperature. After the IAA was removed, 200 µl of acetonitrile was added to dehydrate the gel pieces for 10 min at room temperature. Acetonitrile was then removed and 200 µl of 100 mM ammonium bicarbonate was added to rehydrate the gel pieces for 10 min. Upon the removal of the ammonium bicarbonate, 200 µl of acetonitrile was added to dehydrate the gel pieces. After 10-min incubation time, acetonitrile was removed and the gel pieces were dried by vacuum centrifugation for

5 min at room temperature. To carry out trypsin digestion, 30 µl of freshly prepared trypsin solution (Section 2.13.9) was added to the dried gel pieces and incubated for 30 min on ice with occasional vortex mixing. Excess trypsin solution was removed by centrifugation. An additional 5 µl of 50 mM ammonium bicarbonate was added to the gel pieces prior to the overnight trypsin digestion which was carried out at 37 °C.

2.8.2 Peptide extraction

After an overnight in-gel trypsin digestion, 30 µl of 50 mM ammonium bicarbonate (Section 2.13.9) was added to the gel pieces and incubated for 10 min in an ultrasonic bath with occasional gentle vortex mixing at room temperature. The gel pieces were collected by centrifugation and the supernatant was transferred to a fresh tube for collecting digested peptides. A volume of 30 µl of extraction buffer (Section 2.13.9) was added to the gel pieces for incubating for 10 min in an ultrasonic bath with occasional gentle vortex mixing again at room temperature. The supernatant was collected using the same tube. The step of adding 30 µl of extraction buffer for 10-min incubation in an ultrasonic bath was repeated twice and the supernatant was collected using the same tube. Lastly, 20 µl of acetonitrile was added to the gel pieces and incubated for 10 min in an ultrasonic bath with

occasional gentle vortex mixing at room temperature. The gel pieces were spun down and acetonitrile was then collected to the same tube for collecting digested peptides. The digested peptides were dried by vacuum centrifugation for 3 hr at room temperature.

2.8.3 Desalting of digested peptides

The pellets of digested peptides were dissolved in 10 μl of 0.1% (v/v) trifluoroacetic acid (TFA; Section 2.13.9). The 10-μl sample solution was filtered by Ziptip-C18 (Millipore) for desalting and concentrating the sample. The Ziptip was firstly equilibrated with 10 μl of 50% (v/v) acetonitrile (Section 2.13.9) twice, and then by 10 μl of 0.1% (v/v) TFA twice. The peptides were filtered through the Ziptip repeatedly for 20 cycles. The Ziptip was then washed with 0.1% (v/v) TFA for 5 times, and then the bound peptides were eluted into 2 μl of elution buffer (Section 2.13.9) by passing the elution solution through the Ziptip for 5 cycles. The eluent was collected in a fresh 0.5-ml microfuge tube.

2.8.4 Mass spectrometric analysis of protein candidates

A volume of 0.5 μl of the desalted peptides was dotted onto a MALDI plate (Applied Biosystems). After the peptides were dried, 0.5 μl of matrix solution

(Section 2.13.9) was dotted over the peptide spots. After the matrix solution was dried, the samples were subjected to mass spectrometric analysis. Tandem mass spectrometric (MS/MS) data were acquired using a 4700 Proteomics Analyzer (Applied Biosystems) with TOF/TOF. The data was queried against the National Center for Biotechnology Information (NCBI) database using the MASCOT program (Matrix Sciences) run by the GPS Explorer TM Software v3.7 (Applied Biosystems).

2.9 Electrotransfer and Western blotting

The SDS-PAGE gel (Section 2.6) was equilibrated with 1X transfer buffer (Section 2.13.10). Electrotransfer was carried out by Mini Trans-Blot or Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories). Polyvinylidene fluoride (PVDF) membrane (PALL) was first activated by immersing in analytical-graded methanol for a few seconds, and then equilibrated in 1X transfer buffer. Four pieces of 3-mm filter papers (Whatman) and 2 fiber pads were also soaked in 1X transfer buffer. The gel and membrane were assembled at the centre of the transfer gel holder cassette with the gel placed near the cathode, and was sandwiched by 4 pieces of filter papers and then 2 filter pads by the cassette. The sandwich was then rolled over using a glass tube to expel any trapped air bubbles. The cassette was then assembled and placed into the transfer tank. A frozen Bio-Ice cooling unit was

also placed into the tank of Mini Trans-Blot system whereas the whole tank of Trans-Blot system was placed inside an ice box for cooling purpose. The tank was filled with 1X transfer buffer and electrotransfer was performed at constant voltage of 80 V (Mini Trans-Blot) or 100 V (Trans-Blot) for 2 hr.

After the transfer, the PDVF membrane was taken out from the transfer gel holder cassette, washed in 1X Tris-Buffered Saline (TBS; Section 2.13.10) briefly, and blocked by blocking buffer (Section 2.13.10) for 1 hr at room temperature with gentle shaking. The membrane was then incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Primary antibodies used were rabbit polyclonal anti-DBE 72T (1:1,000; Chan et al., 2001) and rabbit polyclonal anti-histone H3 (1:2,000; Abcam). After the incubation with primary antibodies, the membrane was washed four times in Tris-Buffered Saline-Tween-20 (TBS-T; Section 2.13.10) each of 15 min at room temperature, followed by the incubation with secondary antibodies diluted in blocking buffer for 2 hr at room temperature. The membrane was further washed four times in TBS-T each of 15 min at room temperature before signal detection using Enhanced Chemiluminescent (ECL) Western blotting detection reagents (Amersham Biosciences). One volume of ECL reagent 1 and one volume of ECL reagent 2 were mixed and applied onto the

membrane, and incubated for 1 min at room temperature. The membrane was then wrapped with a plastic wrap and used to expose an X-ray film (FUJI super RX) at desired time intervals. The film was developed by a medical X-ray film processor (Kodak). For re-probing the membranes with other antibodies, Restore Western Blot Stripping Buffer (Pierce) was applied to the membrane, and the membrane was incubated in the stripping buffer for 10 min at room temperature with gentle shaking to remove the primary and secondary antibodies from the membranes. Membranes were washed with TBS-T for 5 min twice prior to re-probing with other antibodies.

2.10 Sucrose gradient sedimentation

Two hundred fly heads (Section 2.3.2) were homogenized in a pre-chilled mortar and pestle. A volume of 1 ml of ice-cold sample lysis buffer (Section 2.13.11) freshly supplemented with 1 mM PMSF was added to the mortar to homogenize fly head samples. Fly head extracts were then transferred to a 1-ml Dounce homogenizer (Wheaton) for further homogenization on ice with 20 loose strokes and 50 tight strokes. Homogenates were transferred to a 1.5-ml microfuge tube for centrifugation at 16,100 X g for 5 min at 4°C twice to clear cell debris. To generate a stepwise gradient, 2.1 ml of each ice-cold sucrose gradient buffer of different sucrose concentrations (10%; 20%; 30%; 40%; 50%; w/v; Section 2.13.11)

freshly supplemented with 0.5 mM PMSF was loaded sequentially from the bottom to the top of an ultra-clear centrifuge tube (Beckman) through a thin capillary tube connected to a 1-ml syringe. The stepwise gradient was left overnight at 4°C to give a continuous gradient. A volume of 900 µl of the fly head homogenates was mixed with 100 µl of 50% (w/v) sucrose gradient buffer to give a final 5% (w/v) sucrose in the sample before overlaying the sample gently onto the continuous sucrose gradient. The ultra-clear centrifuge tube was centrifuged at 151,263 X g (Beckman XL-100K ultracentrifuge; SW 41 Ti rotor) for 16 hr at 4°C. For fraction collection, the ultra-clear centrifuge tube was held upright by a clamp stand. A tiny hole was pierced at the bottom of the tube using a 25G fine needle for solution to drip out. Each fraction of approximately 0.5 ml was collected in a 1.5-ml microfuge tube and was subjected to TCA precipitation (Section 2.5), SDS-PAGE (Section 2.6) and Western blot analysis (Section 2.9).

2.11 Glutathione S-transferase (GST) pull-down assay

2.11.1 Construction of pRSETA-GST-*rps9* plasmid

2.11.1.1 Total RNA preparation from fly heads

Sixteen fly heads (8 males and 8 females) were decapitulated from 2- to 3-day

old adult flies (Section 2.3.1) and homogenized using a plastic pestle in 800 µl of TRIZOL reagent (Invitrogen). Homogenates were centrifuged at 12,000 X g for 10 min at 4°C. Supernatant was collected and incubated for 5 min at room temperature. A volume of 160 µl of chloroform was added to the supernatant with vigorous vortex mixing. The sample mixture was centrifuged at 12,000 X *g* for 15 min at 4°C. The upper aqueous phase was transferred to a fresh tube. A volume of 400 µl of isopropanol was added to the samples and incubated overnight at -20°C to precipitate the RNA. The sample was centrifuged at 12,000 X *g* for 15 min at 4°C. The pellets were washed with 1 ml of 70% (v/v) ethanol twice before dissolving in 50 µl of diethyl pyrocarbonate (DEPC)-treated water. The RNA extracted was stored at -80°C.

2.11.1.2 DNase treatment on extracted RNA

Ribonuclease (RNase)-free deoxyribonuclease (DNase; Promega) was used to remove residual deoxyribonucleic acid (DNA) in the extracted RNA. A reaction mixture of 50 µl of total RNA, 6 µl of 10X DNase buffer (Promega), 3 µl of DNase (1 U/µl; Promega) and 1 µl of DEPC-treated water was incubated for 15 min at room temperature. To terminate the reaction, 6 µl of DNase stop solution (Promega) was added to the reaction mixture and incubated for 10 min at 65°C. To precipitate

RNA, 8 µl of sodium acetate and 6 µl of DEPC-treated water were added to the reaction mixture followed by the addition of 160 µl of 100% ethanol. The reaction mixture was stored overnight at -80°C. The sample was centrifuged at 12,000 X g for 15 min at 4°C to obtain RNA pellet. Ethanol was removed and the RNA pellet was washed in 1 ml of 70 % (v/v) ethanol, and the sample was centrifuged again at 12,000 X g for 10 min at 4°C. Ethanol was then discarded and the pellet was air-dried at room temperature, and redissolved in 50 µl of DEPC-treated water.

2.11.1.3 Reverse Transcription

Reverse transcription was performed by mixing 1 µl of oligo-dT primer (50 µM; Roche) and 4 µl of the extracted RNA. The samples were heated at 70°C for 5 min and then incubated for 5 min at 4°C. The RNA was added the reaction mix containing 4 µl of 5X reaction buffer (Promega), 2.4 µl of Magnesium Chloride ($MgCl_2$; 25 mM) (Promega), 0.5 µl of dNTPs mix (20 mM; GE Bioscience), 0.5 µl of recombinant ribonuclease inhibitor (40 U/µl; Invitrogen) and 1 µl of ImPromII™ reverse transcriptase (Promega), diluted with 6 µl of DEPC-treated water. The RT reaction was performed as follows: 5 min at 25°C, 60 min at 42°C, and 15 min at 70°C to obtain complementary DNA (cDNA).

2.11.1.4 PCR amplification of DNA fragment for cloning

To make a construct for expressing GST-RpS9 protein, *rps9* cDNA was first cloned into a GST-containing expression vector pRSETA-GST-EK (a kind gift of Professor K. B. Wong, CUHK) and then subcloned into another GST-containing expression vector pGEX-6p-1 (Amersham Biosciences). To facilitate the insertion of *rps9* cDNA into the pRSETA-GST-EK vector, a forward primer (*Kpn*I *rps9*) 5'-ggg gta ccg gat ggt gaa cgg ccg cat ac-3' which contains a *Kpn*I restriction site, and a reverse primer (*EcoR*I Stop *rps9*) 5'- gga att cta tta gtc ctc ctc ctc ttc agc agc-3'which contains an *EcoR*I restriction site were designed. To amplify the *rps9* open-reading frame, 3 μl of cDNA mix prepared from *Drosophila* S2 cells was added to a reaction mix containing 0.5 μl of *Pfu* DNA polymerase enzyme (Promega; 2-3 U/μl), 5 μl of 10X PCR buffer, 1 μl of forward primer (10 μM), 1 μl of reverse primer (10 μM), 0.5 μl of dNTPs mix (20 mM) and 39 μl of autoclaved double distilled water. PCR conditions were as follows: an initial denaturation step at 95°C for 5 min, followed by 30 cycles with denaturation at 95°C for 30 sec, annealing at 50°C for 45 sec and extension at 72°C for 50 sec. The resulting PCR products were resolved on a 1% (w/v) agarose gel (Section 2.11.16) and visualized under UV fluorescence. A predicted *rps9* PCR product of 607 bp was observed and gel-purified (Biogene, GeneClean). The purified *rps9* PCR product and

pRSETA-GST-EK plasmid were both double-digested with *Kpn*I and *EcoR*I, and resolved on a 1% (w/v) agarose gel. After purification of the digested DNA product from the gel, the *rps9* cDNA insert was ligated into the pRSETA-GST-EK vector using 1 µl of T4 DNA ligase (New England Biolab) for overnight at 16°C. Competent *E. coli* DH5α cells were transformed with the ligation product and clones were selected using LB agar plates containing 100 µg/ml ampicillin (Section 2.13.1). A positive clone containing the 607 bp *rps9* cDNA was allowed to grow overnight in LB liquid medium (Section 2.13.1) containing 100 µg/ml ampicillin (Section 2.13.1) at 37°C. The pRSETA-GST-*rps9* construct was harvested using plasmid DNA minipreps kit (Qiagen). Identity of the construct was verified by DNA sequencing at Macrogen, Inc (Seoul, Korea). The *rps9* cDNA was then subcloned into a pGEX-6p-1 vector through the *BamH*I and *EcoR*I restriction sites. Both pRSETA-GST-*rps9* and pGEX-6p-1 were double-digested with *BamH*I and *EcoR*I and separated by electrophoresis. After purification of the digested vector and insert from the gel, the *rps9* cDNA was ligated to pGEX-6p-1. Ligated product was used to transform competent *E. coli* DH5α. Bacterial cells with the positive clone were allowed to grow overnight in LB medium containing 100 µg/ml ampicillin at 37°C. The pGEX-6p-1-*rps9* construct was harvested by plasmid DNA minipreps kit (Qiagen).

2.11.1.5 Agarose gel electrophoresis

Electrophoresis was carried out by wide-mini-sub cell GT systems (Bio-Rad Laboratories). DNA sample was mixed with 6X DNA loading dye (Section 2.13.12) in volume ratio of 5:1, and run on 1% (w/v) agarose gel in 1X Tris-Borate-EDTA (TBE) electrophoresis buffer (Section 2.13.12) containing 10 mg/ml ethidium bromide at 120 V until the dye front reached two-third of the gel or as appropriate. For each agarose gel, 2 µl of working solution of DNA ladder (Section 2.13.12) was loaded as molecular markers.

2.11.2 Overexpression of GST and GST-RpS9 proteins

To overexpress GST and GST-RpS9 proteins, the pGEX and pGEX-6p-1-*rps9* plasmids were used respectively to transform *E. coli* Rosetta2 (DE3) cells. Transformed clones were used to inoculate 0.5 ml of LB liquid medium (Section 2.13.1) containing 100 µg/ml ampicillin (Section 2.13.1), 30 µg/ml chloramphenicol (USB) and 2% (w/v) glucose. To obtain a starter culture, cells were grown for approximately 4 hr at 37°C. A volume of 1 ml starter culture was then used to inoculate 100 ml of fresh LB medium containing 100 µg/ml ampicillin and 30 µg/ml chloramphenicol. The cells were grown at 37°C at 250 rpm until OD_{600} reached 0.4

to 0.6 unit. Induction of protein expression was initiated by adding 50 µl of 0.4 M IPTG (0.2 mM; Section 2.13.1) to the culture and cells were grown for 3 hr at 37°C. Cells were then harvested by centrifugation at 3,700 X g (Beckman rotor, JLA 16.25) for 10 min at 4°C. The bacterial pellet was resuspended in 1X PBS pH 7.4 (Section 2.13.14) containing 0.5 mM PMSF and 0.1% (v/v) β-mercaptoethanol (4 ml of 1X PBS per 100 ml culture). The cell suspension was sonicated on ice for ten 5-sec cycles with 5 sec break between each cycle. To obtain a soluble protein fraction, the bacterial cell lysates were centrifuged at 12,000 X g for 30 min at 4°C. The supernatant was collected for protein binding in the GST pull-down assay.

2.11.3 GST pull-down assay

GST pull-down assay was performed by immobilizing bacterially expressed GST and GST-RpS9 proteins onto Glutathione Sepharose 4 Fast Flow beads (Amersham Biosciences). All centrifugation steps throughout the experiment were performed at 800 X g for 3 min at 4°C. A volume of 100 µl of glutathione-Sepharose 4 Fast Flow beads was added to a 1.5-ml microfuge tube and was pre-washed with 1 ml of 1X PBS pH 7.4 (Section 2.13.14) for 3 times. Then, 1 ml of soluble bacterial lysates with overexpression of GST or GST-RpS9 (Section 2.11.2) was respectively added to the beads. The tube for protein immobilization

was incubated for 1 hr at 4°C with end-over-end rotation. After the incubation, the beads were collected by centrifugation and washed five times with 1 ml of 1X PBS pH 7.4. The GST- or GST-RpS9-bound beads were then incubated with 35 μg purified DBE protein (Section 2.1.3) in 1 ml of 1X PBS pH 7.4 for 1 hr at 4°C with end-over-end rotation. The beads were then collected by centrifugation and washed with 1 ml of 1X PBS pH 7.4 for 5 times to remove all unbound DBE protein. The specifically bound proteins were eluted by heating the beads for 5 min at 99°C in 40 μl of 6X SDS sample buffer (Section 2.13.7). Protein eluent was subjected to SDS-PAGE (Section 2.13.7) and Coomassie Blue staining (Section 2.13.8).

2.12 Immunoprecipitation of FLAG-DBE in *Drosophila*

A volume of 40 μl of EZview Red Anti-FLAG M2 affinity gel beads (Sigma) was added to a 1.5-ml microfuge tube and washed twice with 500 μl of wash buffer (Section 2.13.13). Fly head protein extracts (Section 2.3.2) were prepared by homogenizing 50 fly heads in 500 μl ice-cold lysis buffer (Section 2.13.13). The homogenate was centrifuged at 16,100 X g for 1 min at 4°C to clear cell debris. The fly protein extracts were then incubated with the washed EZview Red Anti-FLAG M2 affinity gel beads overnight at 4°C with end-over-end rotation. The gel beads were collected by centrifugation at 8,200 X g for 1 min at 4°C, and washed

with 500 µl of ice-cold lysis buffer for 5 times. The specifically bound proteins were eluted by heating the beads for 5 min at 99°C in 30 µl of 6X SDS sample buffer (Section 2.13.7). Protein eluent was subjected to SDS-PAGE (Section 2.6) and Coomassie Blue staining (Section 2.7).

2.13 Reagents and buffers

2.13.1 Bacterial culture medium

Ampicillin

The stock solution was prepared by dissolving ampicillin in double distilled water to give a final concentration of 100 mg/ml. It was filtered using a 0.22-µm filter (Millipore) for sterilization. The stock solution was kept at -20°C.

Luria-Bertani (LB) agar plate containing 100 µg/ml ampicillin

One litre of LB agar solution was prepared by dissolving 20 g of LB broth powder (USB) and 15 g of bacto-agar (USB) in double distilled water and made up to 1 litre. It was sterilized by autoclave and cooled down before adding 10 ml of 100 mg/ml ampicillin. Each LB agar plate was made by pouring 10 ml of LB agar

solution into a 90-mm Petri dish and stored at 4°C.

LB medium

One litre LB medium was prepared by dissolving 20 g of LB broth powder (USB) and made up to 1 litre with double distilled water. The medium was sterilized by autoclave and it was stored at room temperature.

Isopropyl β-D-thiogalactopyranoside (IPTG)

The stock solution was prepared by dissolving IPTG (USB) in double distilled water to give a final concentration of 0.4 M. The stock solution was stored at –20°C.

2.13.2 Buffers for purification of DBE protein in *Escherichia coli*

200 mM Monobasic sodium phosphate

One litre of 200 mM monobasic sodium phosphate (USB) was prepared by dissolving 27.6 g monobasic phosphate in double distilled water and made up to 1 litre.

200 mM Dibasic sodium phosphate

One litre of 200 mM dibasic phosphate (USB) was prepared by dissolving 53.61 g dibasic phosphate in double distilled water and made up to 1 litre.

Buffer A

One litre of buffer A (50 mM sodium phosphate buffer; pH 7.4) was prepared by mixing 47.5 ml of 200 mM monobasic sodium phosphate and 202.5 ml of 200 mM dibasic sodium phosphate and then made up to one litre by double distilled water. Buffer was filtered using a 0.22-μm filter (Millipore) before use. It was stored at room temperature.

Buffer B

One litre of buffer B (1 M NaCl, 50 mM sodium phosphate buffer; pH 7.4) was prepared by dissolving 58.5 g of NaCl in buffer A and made up to one litre by buffer A. Buffer was filtered using a 0.22-μm filter (Millipore) before use. It was stored at room temperature.

Elution buffer

It was prepared by mixing 4 volumes of buffer A and 1 volume of buffer B. It

was stored at room temperature.

2.13.3 Reagent for preparing protein lysates from *Drosophila* cultured cells

Hypotonic lysis buffer

The buffer was prepared by mixing 1 ml of 1 M Tris-hydrochloric acid (Tris-HCl); pH 7.4, 1 ml of 1 M NaCl, 3 ml of 0.3 M $MgCl_2$ and 0.5 ml of Nonidet P-40 (NP-40; 0.5%, v/v) in a final volume of 100 ml of double distilled water. It was kept at 4°C. One percent (v/v) protease inhibitor cocktail (Sigma) was added freshly to the hypotonic lysis buffer before use.

2.13.4 Reagents for *Drosophila* culture and genetics

Drosophila strains

Transgenic fly strains used in this study include *gmr-GAL4*, UAS-*dbe* and UAS-FLAG-*dbe*. Detailed information of fly stocks is summarized in Table 2.1.

Table 2.1. Summary of *Drosophila* strains used.

Transgenic line	Stock Number	Genotype	Chromosomal location	Description	Reference
gmr-GAL4	LDR8	*w; gmr-GAL4*	X; 2	*glass multiple reporter* (*gmr*)-*GAL4* is a promoter-GAL4 line for ectopic GAL4 expression in all eye cells posterior to the morphological furrow	Ellis et al. (1993). *Development* **119**, 855-865.
dbe	LDR49	*y[1]w; UAS-dbe(1373)/TM3,Sb*	X; 3	for ectopic expression of Dribble (DBE) protein	Chan et al. (2001). *Mol Biol Cell* **12**, 1409-1419.
FLAG-*dbe*	LDR51	*y[1]w; UAS-FLAG-dbe(1429)/TM3,Sb*	X; 3	for ectopic expression of FLAG-DBE protein	Chan et al. (2001). *Mol Biol Cell* **12**, 1409-1419.

Cornmeal-yeast-glucose-agar medium

It was prepared by dissolving 12.5 g agar (1.25%, w/v), 105 g dextrose (10.5%, w/v), 105 g maize (10.5%, w/v), and 21 g yeast (2.1%, w/v) in distilled water. After boiling the mixture for about 20 min, 80 ml of 1% (w/v) Nipagen (methyl p-hydroxybenzoate; Sigma) in ethanol was supplemented as mold inhibitor and then made up to 1 litre by distilled water. Aliquots of 15 ml fly culture medium were added into each plastic culture vial.

2.13.5 Buffers for immobilization of proteins onto AminoLink Plus coupling gel

Coupling buffer

The Coupling buffer (Pierce) is a 0.1 M sodium phosphate and 0.15 M NaCl at pH 7.4 supplemented with 0.05% (w/v) sodium azide (NaN_3). It was kept at 4°C.

Quenching buffer

The quenching buffer (Pierce) is a 1 M Tris-HCl supplemented with 0.05% (w/v) NaN_3 at pH 7.4. It was kept at 4°C.

Washing solution

The washing solution was prepared by dissolving NaCl in double distilled water to give a final concentration of 1 M and the solution was supplemented with 0.05% (w/v) NaN_3. It was kept at 4°C.

2.13.6 Buffers for affinity pull-down using protein coupled-beads

Washing buffer

The washing buffer was prepared by dissolving NaCl in buffer A (Section 2.13.2) to give a final concentration of 250 mM NaCl. It was kept at 4°C.

Elution buffer

The elution buffer was prepared by dissolving NaCl in buffer A (Section 2.13.2) to give a final concentration of 1 M NaCl. It was kept at 4°C.

2.13.7 Reagents for SDS-PAGE

SDS sample buffer

SDS sample buffer (6X) was prepared by mixing 10 mg of bromophenol blue

(0.02%, w/v), 5 ml of 1 M Tris-HCl; pH 6.8 (0.1 M), 10 ml of 10% (w/v) SDS (2 %, w/v), 10 ml of glycerol (20%, v/v), and 2.5 ml of β-mercaptoethanol (Sigma) (5%, v/v) to a final volume of 50 ml with double distilled water. Aliquots of 1.5 ml were stored at -20°C.

SDS electrophoresis buffer

Stock SDS electrophoresis buffer (10X) was prepared by dissolving 30.28 g of Tris-base (0.25 M), 144.13 g of glycine (1.92 M), and 10 g of SDS (1%, w/v) in a final volume of 1 litre with double distilled water; pH was adjusted to 8.3. Working SDS electrophoresis buffer (1X) was prepared by diluting the stock buffer (10X) by 10-fold with double distilled water. Both stock and working solutions were kept at room temperature.

Separating gel solution

For each mini-gel of 1.5-mm spacers, separating gel solution was prepared by mixing 3.5 ml of 30% acrylamide (12%, v/v; BioRad), 3.255 ml of 1 M Tris-HCl; pH 8.8 (0.4 M), 1.9 ml of double distilled water, 87.5 µl of 10% (w/v) SDS (1%, w/v), 35 µl of 10% (w/v) ammonium persulfate (APS; 0.04%; w/v), and 4 µl of N,N,N',N'-Di-(dimethylamino)ethane (TEMED).

For each 16 x 20 cm gel of 1-mm spacers, separating gel solution was prepared by mixing 12.5 ml of 30 % acrylamide (12%, v/v), 11.62 ml of 1 M Tris-HCl; pH 8.8 (0.4 M), 6.78 ml of double distilled water, 312.4 µl of 10% (w/v) SDS (1%, w/v), 125 µl of 10% (w/v), APS (0.04%; w/v), and 14 µl of TEMED.

Stacking gel solution

For each mini-gel of 1.5-mm spacers, stacking gel solution was prepared by mixing 0.49 ml of 30% acrylamide (4%, v/v), 0.966 ml of 0.1 M Tris-HCl; pH 6.8 (0.03 M), 1.96 ml of double distilled water, 35 µl of 10% (w/v) SDS (1%, w/v), 14 µl of 10% (w/v), APS (0.04%; w/v), and 4 µl of TEMED.

For each 16 x 20 cm gel of 1-mm spacers, stacking gel solution was prepared by mixing 1.4 ml of 30% acrylamide (4%, v/v), 2.7 ml of 0.1 M Tris-HCl; pH 6.8 (0.03 M), 5.5 ml of double distilled water, 98 µl of 10% (w/v) SDS (1%, w/v), 39 µl of 10% (w/v), APS (0.04%; w/v), and 11 µl of TEMED.

2.13.8 Reagent for Coomassie Blue Staining and Destaining

Coomassie Blue staining solution

It was prepared by mixing 0.5 g of Coomassie R-250 (0.5%, w/v), 10 ml of acetic acid (10%, v/v), 45 ml of ethanol (45%, v/v) and made up to a final volume of 100 ml with double distilled water. The mixture was stored at room temperature.

Destaining solution

It was prepared by mixing 100 ml acetic acid (10%, v/v), 400 ml of ethanol (40%, v/v) and made up to a final volume of 1 litre with double distilled water. The mixture was stored at room temperature.

2.13.9 Reagents for protein identification by tandem mass spectrometry

100 mM Ammonium bicarbonate

It was prepared by dissolving 0.2 g of ammonium bicarbonate (100 mM) in a final volume of 20 ml with double distilled water. It was stored at room temperature.

50 mM Ammonium bicarbonate

It was prepared by mixing one volume of 100 mM ammonium bicarbonate (50

mM) with one volume of double distilled water. It was stored at room temperature.

10 mM Dithiothreitol

It was freshly prepared by dissolving 1.5 mg of DTT (10 mM) in 1 ml of 100 mM ammonium bicarbonate.

100 mM Iodoacetamide

It was freshly prepared by dissolving 18 mg of iodoacetamide (100 mM) in 1 ml of 100 mM ammonium bicarbonate.

Trypsin solution (20 ng/µl)

It was freshly prepared by adding 1 ml of ice-cold 50 mM ammonium bicarbonate to 20 µg of sequencing-grade modified trypsin (20 ng/µl; Promega). It was kept on ice until use.

Extraction buffer

It was freshly prepared by mixing 10 ml of acetonitrile (50%; v/v), 1 ml of trifluoroacetic acid (TFA; 5%; v/v; Sigma) and made up to 20 ml using double distilled water.

0.1% (v/v) Trifluoroacetic acid (TFA)

It was freshly prepared by adding 10 µl of TFA (0.1%; v/v) to 10 ml of double distilled water.

50% (v/v) Acetonitrile

It was prepared by mixing 25 ml of acetonitrile (50%; v/v; Sigma) with double distilled water and made up to a final volume of 50 ml. It was stored at room temperature.

Eution buffer

It was prepared by mixing 10 ml of acetonitrile (50%; v/v), 1 ml of TFA (5%; v/v) and made up to a final volume of 20 ml using double distilled water.

Matrix solution

It was freshly prepared by dissolving 10 mg of ACH-Cinnamic acid (1%; w/v; Amersham Biosciences) in 1 ml of elution buffer.

2.13.10 Reagents for electrotransfer and Western blotting

Transfer buffer

Stock transfer buffer (10X) was prepared by dissolving 30.28 g of Tris-base (0.25 M) and 144.13 g of glycine (1.92 M) in a final volume of 1 litre double distilled water. Stock solution was kept at room temperature. Working transfer buffer (1X) was freshly prepared by mixing 70 ml of the stock transfer buffer (10X) and 70 ml of methanol to a final volume of 700 ml with double distilled water.

Tris-buffered saline (TBS)

Stock TBS buffer (10X) was prepared by dissolving 24.22 g of Tris-base (0.2 M) and 80.06 g of NaCl (1.37 M) in a final volume of 1 litre with double distilled water; pH was adjusted to 7.6. Working TBS buffer (1X) was prepared by diluting the stock solution by 10-fold with double distilled water. Both stock and working solutions were kept at room temperature.

Tris-buffered saline- Tween-20 (TBS-T)

TBS-T solution was prepared by adding 0.5 ml of Tween-20 (0.05 %, v/v) and to a final volume of 1litre with 1X TBS. It was kept at room temperature.

Blocking buffer

Blocking buffer was prepared by dissolving 5 g of non-fat milk powder (5%, w/v) in 100 ml of 1X TBS. It was kept for a week at 4°C.

2.13.11 Reagents for sucrose gradient sedimentation

100 mM HEPES; pH 7.5

It was prepared by dissolving 2.383 g of N-[2-Hydroxyethyl] piperazine-N'-[2-Ethanesulfonic Acid] (HEPES) in a final volume of 100 ml of double distilled water; pH was adjusted to 7.6.

Sample buffer

Sample buffer was prepared by mixing 20 ml of 100 mM HEPES; pH 7.5 (20 mM), 10 ml of 100 mM potassium chloride (KCl; 10 mM), 5 ml of 50 mM $MgCl_2$ (2.5mM), 1 ml of 100 mM EGTA; pH 11 (1 mM) in a final volume of 100 ml of double distilled water. It was kept at 4°C.

Sucrose gradient buffers

Sucrose gradient buffers were prepared by dissolving different concentrations of sucrose (10%; 20%; 30%; 40%; 50%; w/v) in sample buffer. The buffers were kept at 4°C.

2.13.12 Reagents for agarose gel electrophoresis

Tris-borate-EDTA (TBE) Electrophoresis Buffer (5X)

Stock TBE buffer (5X) was prepared by dissolving 54 g of Tris base (0.45 M), 27.5 g of boric acid (0.45 M) and 20 ml of 0.5 M EDTA; pH 11 in double distilled water to a total volume of 1 litre with pH adjusted to 8.0. Working solution (1X) was prepared by diluting the stock buffer 5-fold in double distilled water. Both stock and working solutions were kept at room temperature.

DNA loading dye (6X)

Loading dye was prepared by mixing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 40% (v/v) glycerol in autoclaved double distilled water. Stock was kept as aliquots of 1 ml at –20°C while the working dye was stored at 4°C.

DNA Ladder Marker

The 100 bp and 1 kbp DNA markers were purchased from Fermentus. The working solution was prepared by mixing 1 volume of stock DNA markers and 1 volume of 6X loading dye with 4 volumes of autoclaved double distilled water. The stock solution was kept at –20°C while the working solution was stored at 4°C. For each agarose gel, 1 µl of working solution was loaded.

2.13.13 Reagents for immunoprecipitation

Wash buffer

Wash buffer was prepared by mixing 0.061 g of Tris-base (50 mM) and 0.088 g of NaCl (0.15 M) in a final volume of 100 ml of double distilled water; pH was adjusted to 7.6. It was kept at 4°C.

Lysis buffer with 1% (v/v) NP-40 and 1% (w/v) SDS

Lysis buffer was prepared by mixing 0.061 g of Tris-base (50 mM), 0.088 g of NaCl (0.15 M), 1 ml of NP-40 (1%, v/v), 1 ml of sodium deoxycholate (1%, v/v), and 10 ml of 10% (w/v) SDS (1%, w/v) in a final volume of 100 ml of double distilled water; pH was adjusted to 7.6. It was kept at 4°C.

Lysis buffer with 1% (v/v) Triton X-100 and 2% (w/v) SDS

Lysis buffer was prepared by mixing 0.061 g of Tris-base (50 mM), 0.088 g of NaCl (0.15 M), 1 ml of Triton X-100 (1%, v/v), 1 ml of sodium deoxycholate (1%, v/v), and 20 ml of 10% (w/v) SDS (2%, w/v) in a final volume of 100 ml of double distilled water; pH was adjusted to 7.6. It was kept at 4°C.

2.13.14 Other common buffer

Phosphate-buffered saline

Stock PBS solution (10X) was prepared by dissolving 80 g of NaCl (1370 mM), 2 g of KCl (27 mM), 14.4 g of dibasic sodium phosphate (100 mM) and 2.4 g of monobasic potassium phosphate (20 mM) in a final volume of 1 litre with double distilled water; pH was adjusted to 7.4. Working PBS solution (1X) was prepared by diluting the stock buffer (10X) by 10-fold with double distilled water. Working solution was sterilized by autoclave. Both stock and working solutions were kept at 4°C.

3. RESULTS

3.1 Identification of DBE protein interactors by affinity pull-down

3.1.1 Introduction:

The role of DBE in rRNA processing was shown by the cleavage defects of rRNA processing in *dbe* mutants (Chan et al., 2001). However, DBE lacks any known enzymatic domains to explain for the underlying cause of the pre-rRNA cleavage defects. It is known that rRNA processing involves successive modifications and cleavages of pre-rRNA at specific sites (Section 1.1.1.1), thus it would require precise positioning of rRNA processing factors onto pre-rRNA. Since DBE carries a RNA-binding KH domain (Section 1.2.1) and its RNA-binding property has been demonstrated *in vitro* (Yiu et al., 2006), it is hypothesized that DBE serves to recruit rRNA processing factors, e.g. nucleases, onto pre-rRNA for cleavage. Therefore, identifying protein interactors of DBE would help to elucidate the role of DBE in rRNA processing.

The main focus of this study is to identify protein interactors of DBE using affinity pull-down experiment. Bacterially expressed DBE protein was

immobilized onto resin beads to generate DBE-coupled beads which serve as a bait to capture DBE-interactors in *Drosophila* cultured cells. Proteins bound onto the DBE-coupled beads were eluted and subjected to SDS-PAGE and mass spectrometric identification. To demonstrate the specificity of DBE-interactors pulled down by DBE-coupled beads, two control experiments which used lysozyme- and heat-denatured DBE-beads were also performed.

3.1.2 Results:

3.1.2.1 Expression and purification of DBE protein in *E. coli*

The method of DBE purification was adapted from Yiu et al. (2006). Since DBE has a high predicted isoelectric point of 9.83 and most bacterial proteins have lower pI values, cation exchange chromatography (CIEX) was chosen as the first step for DBE purification. After overexpressing DBE in *E. coli* C41(DE3), bacterial cells were sonicated in phosphate buffer of pH 7.4 to obtain soluble lysates. Since DBE is positively charged under pH 7.4, it can bind to the cation exchange resins. After the injection of the soluble fraction into the Hi-Trap SP HP cation exchange column, unbound proteins were washed away, and DBE was eluted by increasing the ionic strength of the elution buffer to 50-60 mM NaCl (Figure 3.1.1A).

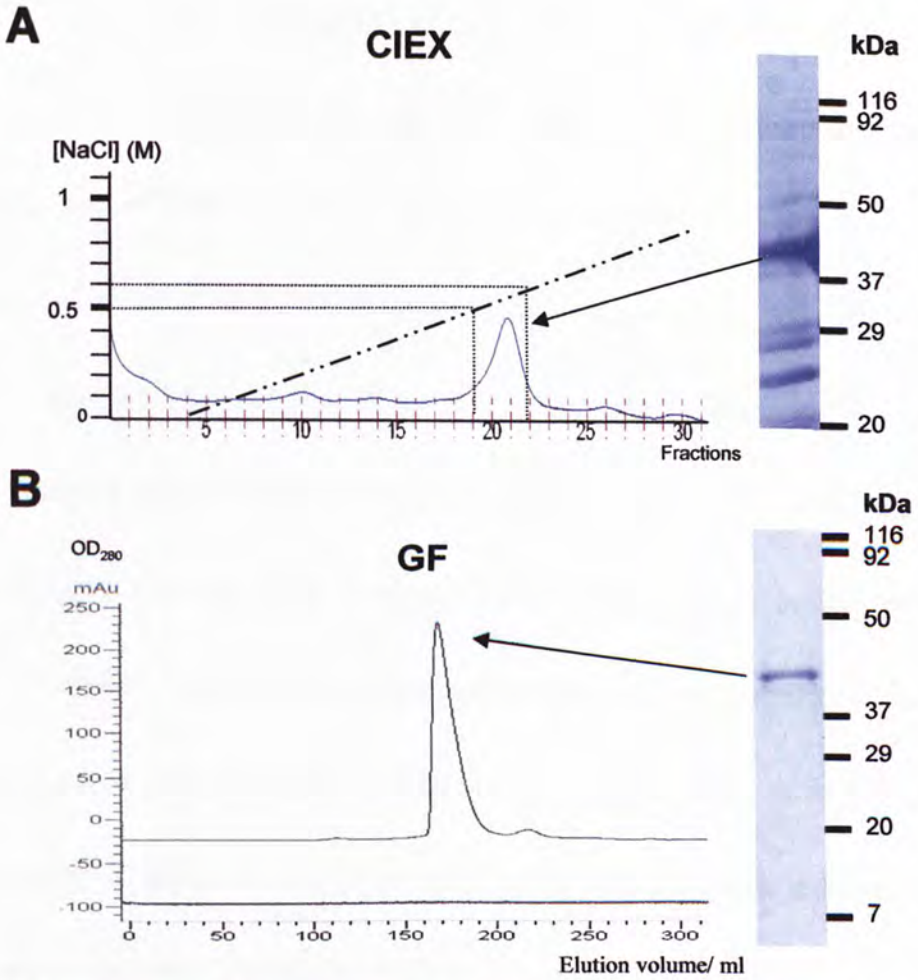


Figure 3.1.1 Purification of bacterially-expressed DBE protein. (A) On the left panel is the elution profile of the cation exchange chromatography (CIEX) for purification of DBE using Hi-Trap SP HP cation exchange column. A linear sodium chloride (NaCl) gradient was used to elute proteins and the gradient is indicated with a line (—··—·). A peak was observed when the NaCl concentration of the elution buffer reached 500 - 600 mM. The 19th to 23rd fractions which corresponded to this peak were pooled and concentrated for gel filtration chromatography (GF). On the right panel is the SDS–PAGE analysis of the peak elution of IEX. **(B)** On the left panel is the elution profile of DBE from the Hi-Load Superdex 200 prep grade column. A peak was observed at approximately 180 ml of elution volume after protein injection. On the right panel is the SDS–PAGE analysis of the peak elution of GF. The purified DBE appeared as a homogeneous band in the SDS–PAGE gel.

The protein fractions corresponding to the peak of OD_{280} absorbance in the CIEX elution profile were subjected to SDS-PAGE analysis. A major protein band of the molecular size of DBE (~40 kDa) and four other contaminant bands which were of smaller molecular sizes (~20 kDa to 29 kDa) were present in the peak elution of CIEX (Figure 3.1.1A). Thus, 19th to 23rd fractions corresponding to the peak in the CIEX elution profile were pooled together and concentrated for the second step of purification with the use of gel filtration chromatography. DBE protein was eluted at 180 ml elution volume after the protein was injected into the Hi-Load Superdex 200 prep grade column (Figure 3.1.1B). The purified DBE after gel filtration chromatography appeared as a single band on SDS-PAGE which indicates that the purified protein was of high purity (Figure 3.1.1B).

3.1.2.2 Affinity pull-down using DBE-coupled beads and the control using lysozyme-coupled beads

As DBE has a predicted isoelectric point of 9.83 which is higher than the pH of the binding condition (pH 7.4) in the affinity pull-down, DBE would be positively charged and thus might interact non-specifically with proteins of opposite charge through electrostatic interactions. To discriminate non-specific electrostatic interactions from the specific ones, lysozyme which also has a high isoelectric point

(pI: 11.3) was used as a control.

Affinity pull-down using DBE- and lysozyme-coupled beads were performed in parallel and their elution patterns were analyzed. The cell-lysate inputs, flow-throughs, washes, final washes and eluents of the affinity pull-down experiments were subjected to SDS-PAGE analysis. The final wash represents the last washing step before eluting proteins that bound to resin beads. Since protein bands that appeared in the eluents were not found in the final wash (Figure 3.1.2), it indicates that protein bands in the eluents did not result from incomplete washing but were eluted due to the increase in ionic strength of the buffer. Eluent of the affinity pull-down using DBE-coupled beads showed approximately 22 bands while for the one using lysozyme-coupled beads showed only 1 protein band (Figure 3.1.2). One of the protein bands appeared in the eluent of affinity pull-down using DBE-coupled beads has a molecular size similar to the only band that was detected in the eluent of affinity pull-down using lysozyme-coupled beads (Figure 3.1.2). Therefore, this protein band was considered to be the result of non-specific protein-protein interaction.

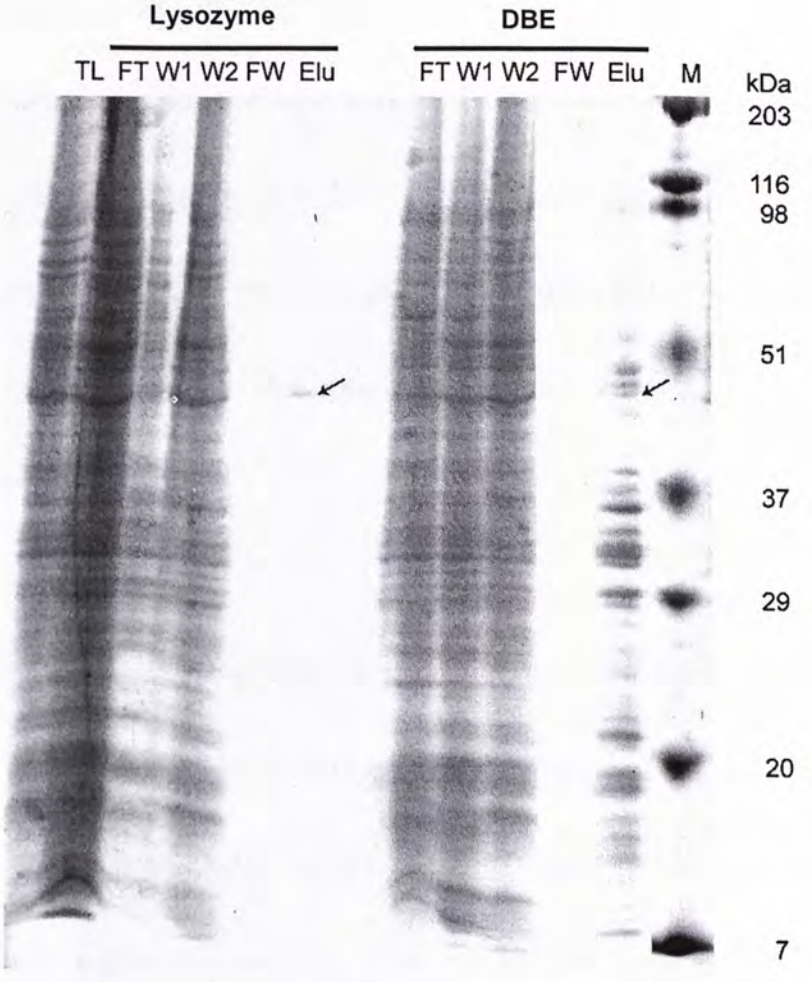


Figure 3.1.2 Elution profile of the affinity pull-down using lysozyme- and DBE-coupled beads. AminoLink Plus coupling resin beads coupled with lysozyme or DBE were incubated with total protein *Drosophila* S2 cell lysates (TL). After incubation, resin beads were washed extensively and unbound proteins were detected in the flowthroughs, washes (W1, W2 and FW). Proteins bound onto the protein-coupled beads were eluted in the eluent (Elu). Each protein fraction from the affinity pull-down experiment was subjected to SDS-PAGE analysis. Eluent of the affinity pull-down with DBE-coupled beads showed approximately 22 protein bands while for the one with lysozyme-coupled beads showed only 1 protein band. Non-specific bands which were pulled down by lysozyme-coupled beads and DBE-coupled beads are indicated with arrows.

3.1.2.3 Affinity pull-down using DBE-coupled beads and the control using heat-denatured DBE-coupled beads

It has been shown that heating causes protein denaturation by altering the native structure of a protein (Furlong et al., 2005; Mirsky and Pauling, 1936). The affinity pull-down experiment using heat-denatured DBE-coupled beads was employed as a control to distinguish proteins that specifically interact with DBE in non-denatured conformation.

Affinity pull-down using DBE- and heat-denatured DBE-coupled beads were performed in parallel and their elution patterns were analyzed. All except two protein bands were not found in the final washes (Figure 3.1.3), indicating that two protein bands might be resulted from incomplete washing of DBE- and heat-denatured DBE-coupled beads after binding. Approximately 20 bands in the eluent of the affinity pull-down using DBE-coupled beads were diminished in intensity or lost in that of heat-denatured DBE control (Figure 3.1.3). The loss or weakening of interactions after DBE had undergone heat denaturation might reflect the dependence of native folding of DBE for protein interactions. Therefore, proteins which interact weakly or did not interact with the heat-denatured DBE control were good indications that these proteins bound specifically to native DBE.

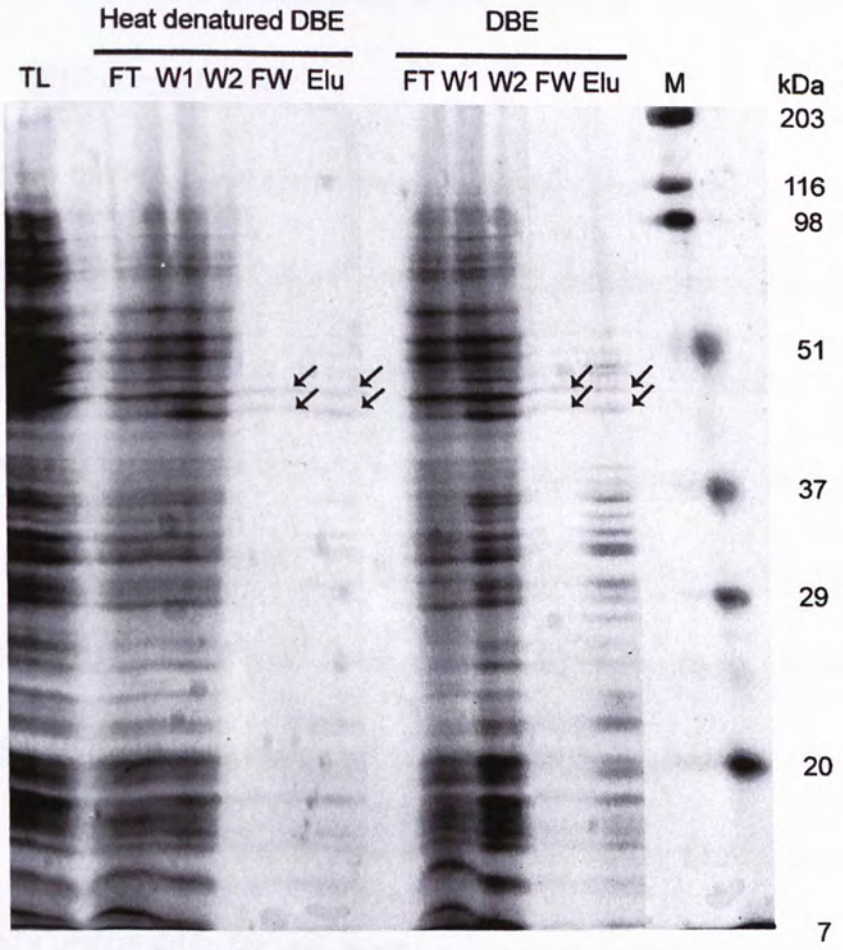


Figure 3.1.3 Elution profile of the affinity pull-down using heat-denatured DBE- and DBE-coupled beads. AminoLink Plus coupling resin beads coupled with heat-denatured DBE or DBE were incubated with total protein *Drosophila* S2 cell lysates (TL). After incubation, resin beads were washed extensively and unbound proteins were detected in the flowthroughs, washes (W1, W2 and FW). Proteins bound onto the protein-coupled beads were eluted in the eluent (Elu). Each protein fraction from the affinity pull-down experiment was subjected to SDS-PAGE analysis. Many proteins bands that appeared in the eluent of the affinity pull-down using DBE-coupled beads disappeared or diminished in the eluent of the control using heat-denatured DBE-coupled beads. Non-specific bands which were pulled down by heat-denatured DBE-coupled beads and DBE-coupled beads are indicated with arrows.

3.1.2.4 Tandem mass spectrometric identification of proteins pulled down by DBE-coupled beads

Before tandem mass spectrometric (MS/MS) identification of proteins bands pulled down by DBE, the elution profiles of the 6 independent affinity pull-down experiments were compared to check the reproducibility of results (Figure 3.1.4). In general, 15 to 24 protein bands were pulled down in each of the 6 independent affinity pull-down experiments; and the overall patterns of the eluents were shown to be reproducible (Figure 3.1.4). Each protein band in the eluents was excised from the SDS-PAGE gels and subjected to in-gel trypsin digestion and subsequently MS/MS analysis. The MS/MS data was analyzed by the Mascot software which generated a protein score for each protein candidate to indicate its level of confidence (Perkins et al., 1999). The protein score reflects the statistical confidence of a match between the MS/MS data and the calculated peptide mass generated after empirical trypsin digestion using the protein sequences deposited in the NCBI database. A score of *p*-value less than 0.05 would be regarded as high confidence. In addition, the MS/MS data was further confirmed by matching the predicted molecular size of the identified protein with the size of the corresponding protein band appeared on the SDS-PAGE (Figure 3.1.5 to Figure 3.1.10).

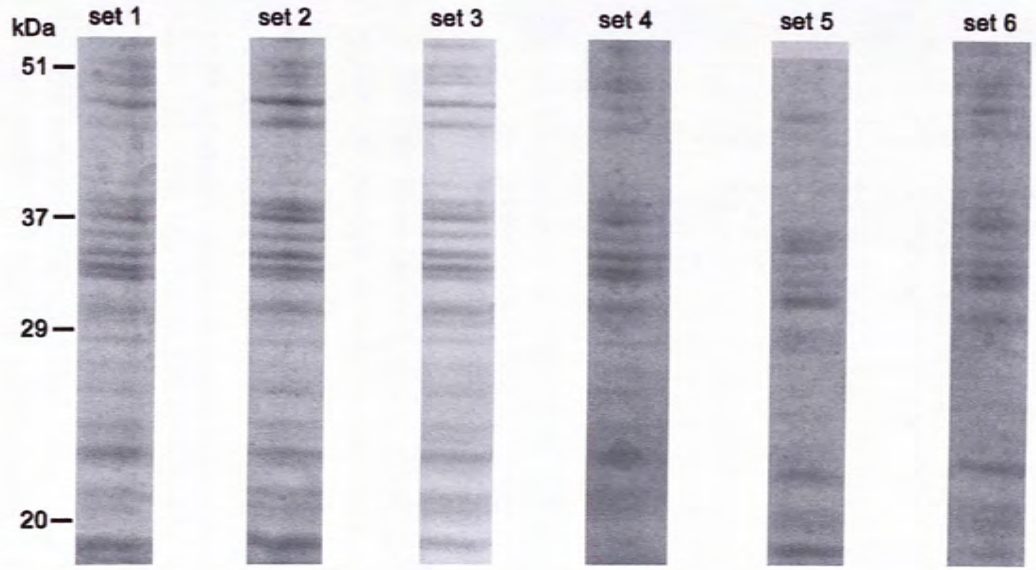
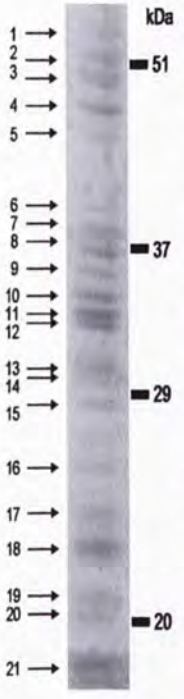
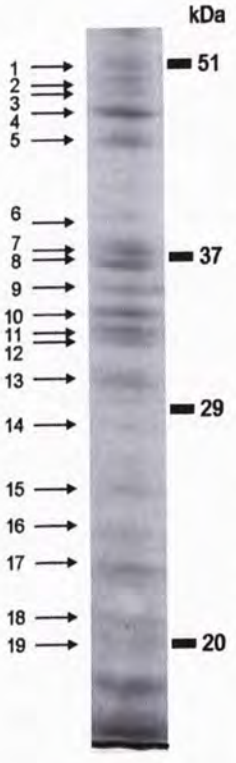


Figure 3.1.4 Comparison of the elution profiles of six sets of the affinity pull-down experiments using DBE-coupled beads. The eluents in the SDS-PAGE gel of the affinity pull-down using DBE-coupled beads were compared. In general, 15 to 24 protein bands were pulled down in each of the 6 independent affinity pull-down experiments. The overall patterns of the eluents were shown to be reproducible.



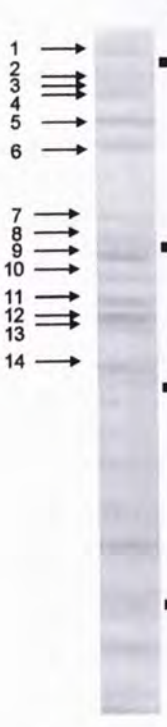
Band no. [a]	DBE-interactor [b]	GI no. [c]	Predicted molecular weight (kDa)	Predicted pI [d]	Protein Score [e]
1	Tubulin	24655741	51.3	4.9	212
2	LP10071p	39752635	50.3	9.14	158
3	Ribosomal protein *L4* (RpL*4*)	24650747	45	11.48	222
4	Actin	156759	41.8	5.37	392
5	Dribble (DBE)	17137346	39.8	9.67	208
6	Ribosomal protein L22 (RpL22)	6249321	31.0	10.11	154
7	Ribosomal protein LP0 (RpLP0)	17737731	34.2	6.48	362
8	Ribosomal protein L23a (RpL23a)	4378006	28.6	10.94	178
8	Ribosomal protein S3a (RpS3a)	17864162	30.3	9.63	92
9	Ribosomal protein L7a (RpL7a)	806545	30.7	10.71	121
10	Not ID				
11	Ribosomal protein L6 (RpL6)	24651668	27.7	10.68	102
12	Ribosomal protein	8484	28.2	10.86	128
13	Ribosomal protein L13 (RpL13)	17647879	24.9	10.99	124
14	Not ID				
15	Not ID				
16	Ribosomal protein S9 (RpS9)	24661711	22.6	10.61	126
16	Ribosomal protein L17 (RpL17)	24640206	21.6	10.31	169
17	Ribosomal protein L14 (RpL14)	1749777	19.2	11.19	186
17	Ribosomal protein L21 (RpL21)	20129705	18.5	10.55	106
18	Not ID				
19	Not ID				
20	Ribosomal protein L35 (RpL35)	24639919	14.4	11.18	96
21	Not ID				

Figure 3.1.5 The first set of tandem mass spectrometric (MS/MS) identification of protein bands in the eluent of the affinity pull-down experiment using DBE-coupled beads. On the left panel is the SDS-PAGE gel of the eluent of the affinity pull-down using DBE-coupled beads. Each excised protein bands for MS/MS analysis are labeled with a number and indicated with an arrow. On the right panel is the table showing the summary of MS/MS results. It depicts the number given to each protein band [a], the name of DBE-interactor identified by MS/MS [b], the GenBank molecular biology database identifier (GI no.) [c], the predicted molecular weight, the predicted isoelectric point (pI) [d] and the protein score which was generated by the Mascot software to show the confidence level of the MS/MS result [e]. Protein bands no. 8, 16 and 17 had two proteins being identified. 'Not ID' denotes the protein was not identified by MS/MS. All the protein scores listed in the table have *p*-values of less than 0.05.



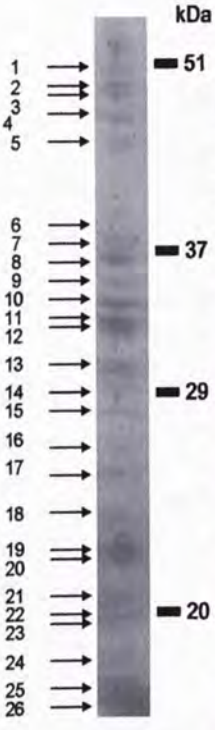
Band no. [a]	DBE-interactor [b]	GI no. [c]	Predicted molecular weight (kDa)	Predicted pI [d]	Protein Score [e]
1	Tubulin	24655741	51.3	4.9	179
2	LP10071p	39752635	50.3	9.14	175
3	Ribosomal protein L4 (RpL4)	24650747	45	11.48	88
4	Actin	156759	41.8	5.37	418
5	Dribble (DBE)	17137346	39.8	9.67	258
6	Ribosomal protein L22 (RpL22)	6249321	31.0	10.11	89
7	Ribosomal protein LP0 (RpLP0)	17737731	34.2	6.48	337
8	Ribosomal protein L23a (RpL23a)	4378006	28.6	10.94	207
8	Ribosomal protein S3a (RpS3a)	17864162	30.3	9.63	87
9	Ribosomal protein L7a (RpL7a)	806545	30.7	10.71	286
10	Not ID				
11	Ribosomal protein L6 (RpL6)	24651668	27.7	10.68	155
12	Ribosomal protein	8484	28.2	10.86	100
13	Not ID				
14	Not ID				
15	Ribosomal protein S9 (RpS9)	24661711	22.6	10.61	66
16	Ribosomal protein L18a (RpL18a)	17647885	21.0	10.62	60
17	Not ID				
18	Ribosomal protein L14 (RpL14)	1749777	19.2	11.19	83
19	Ribosomal protein S15 (RpS15)	20129705	18.5	10.55	81

Figure 3.1.6 The second set of tandem mass spectrometric (MS/MS) identification of protein bands in the eluent of the affinity pull-down experiment using DBE-coupled beads. On the left panel is the SDS-PAGE gel of the eluent of the affinity pull-down using DBE-coupled beads. Each excised protein bands for MS/MS analysis are labeled with a number and indicated with an arrow. On the right panel is the table showing the summary of MS/MS results. It depicts the number given to each protein band [a], the name of DBE-interactor identified by MS/MS [b], the GenBank molecular biology database identifier (GI no.) [c], the predicted molecular weight, the predicted isoelectric point (pI) [d] and the protein score which was generated by the Mascot software to show the confidence level of the MS/MS result [e]. rotein band no. 8 had two proteins being identified. 'Not ID' denotes the protein was not identified by MS/MS. All the protein scores listed in the table have *p*-values of less than 0.05.



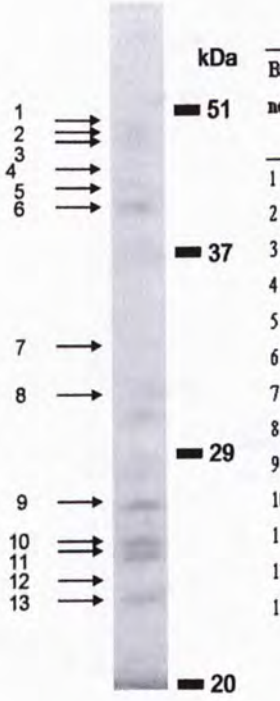
Band no. [a]	DBE-interactor [b]	GI no. [c]	Predicted molecular weight (kDa)	Predicted pI [d]	Protein Score [e]
1	Tubulin	24655741	51.3	4.9	105
2	Not ID				
3	Not ID				
4	LP10071p	39752635	50.3	9.14	253
5	Ribosomal protein *LA* (RpL*A*)	24650747	45	11.48	238
6	Not ID				
7	Not ID				
8	Ribosomal protein LP0 (RpLP0)	17737731	34.2	6.48	349
9	Ribosomal protein L23a (RpL23a)	4378006	28.6	10.94	235
10	Ribosomal protein L7a (RpL7a)	806545	30.7	10.71	104
11	Ribosomal protein L6 (RpL6)	24651668	27.7	10.68	154
12	Ribosomal protein S3 (RpS3)	296094	27.5	9.28	109
13	Ribosomal protein S3a (RpS3a)	17864162	30.3	9.63	89
14	Ribosomal protein	8484	28.2	10.86	82

Figure 3.1.7 The third set of tandem mass spectrometric (MS/MS) identification of protein bands in the eluent of the affinity pull-down experiment using DBE-coupled beads. On the left panel is the SDS-PAGE gel of the eluent of the affinity pull-down using DBE-coupled beads. Each excised protein bands for MS/MS analysis are labeled with a number and indicated with an arrow. On the right panel is the table showing the summary of MS/MS results. It depicts the number given to each protein band [a], the name of DBE-interactor identified by MS/MS [b], the GenBank molecular biology database identifier (GI no.) [c], the predicted molecular weight, the predicted isoelectric point (pI) [d] and the protein score which was generated by the Mascot software to show the confidence level of the MS/MS result [e]. 'Not ID' denotes the protein was not identified by MS/MS. All the protein scores listed in the table have *p*-values of less than 0.05.



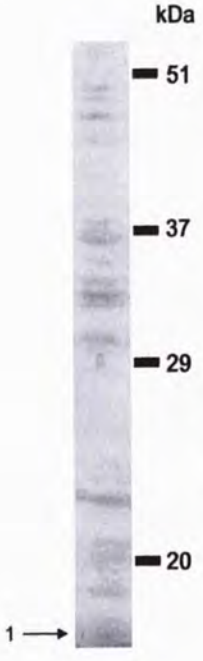
Band no. [a]	DBE-interactor [b]	GI no. [c]	Predicted molecular weight (kDa)	Predicted pI [d]	Protein Score [e]
1	Tubulin	24655741	51.3	4.9	153
2	Ribosomal protein L3 (RpL3)	23170971	46.9	10.25	
3	Ribosomal protein L4 (RpL4)	24650747	45	11.48	281
4	Actin	156759	41.8	5.37	465
5	Dribble (DBE)	17137346	39.8	9.67	256
6	Ribosomal protein L22 (RpL22)	6249321	31.0	10.11	86
7	Ribosomal protein LP0 (RpLP0)	17737731	34.2	6.48	511
8	Ribosomal protein L23a (RpL23a)	4378006	28.6	10.94	273
9	Ribosomal protein S3a (RpS3a)	17864162	30.3	9.63	78
9	Ribosomal protein L7a (RpL7a)	806545	30.7	10.71	464
10	Ribosomal protein L8 (RpL8)	7292251	27.9	11.15	114
11	Ribosomal protein L6 (RpL6)	24651668	27.7	10.68	357
12	Ribosomal protein	8484	28.2	10.86	209
13	Ribosomal protein S8 (RpS8)	23172610	23.7	10.49	203
14	Not ID				
15	Not ID				
16	Not ID				
17	Ribosomal protein S9 (RpS9)	24661711	22.6	10.61	158
18	Ribosomal protein L34b (RpL34b)	23170791	18.4	11.52	120
19	Ribosomal protein L26 (RpL26)	7293850	17.2	10.94	68
20	Ribosomal protein L14 (RpL14)	1749777	19.2	11.19	116
21	Ribosomal protein L12 (RpL12)	7291730	17.7	9.21	291
22	Ribosomal protein L28 (RpL28)	23092892	16.0	11.09	117
23	Ribosomal protein S23 (RpS23)	21645412	16.0	10.59	107
24	Ribosomal protein S16 (RpS16)	7291436	16.7	10.17	88
25	Not ID				
26	Histone H2A	72151624	13.4	10.72	78

Figure 3.1.8 The forth set of tandem mass spectrometric (MS/MS) identification of protein bands in the eluent of the affinity pull-down experiment using DBE-coupled beads. On the left panel is the SDS-PAGE gel of the eluent of the affinity pull-down using DBE-coupled beads. Each excised protein bands for MS/MS analysis are labeled with a number and indicated with an arrow. On the right panel is the table showing the summary of MS/MS results. It depicts the number given to each protein band [a], the name of DBE-interactor identified by MS/MS [b], the GenBank molecular biology database identifier (GI no.) [c], the predicted molecular weight, the predicted isoelectric point (pI) [d] and the protein score which was generated by the Mascot software to show the confidence level of the MS/MS result [e]. Protein band no. 9 had two proteins being identified. 'Not ID' denotes the protein was not identified by MS/MS. All the protein scores listed in the table have *p*-values of less than 0.05.



Band no. [a]	DBE-interactor [b]	GI no. [c]	Predicted molecular weight (kDa)	Predicted pI [d]	Protein Score [e]
1	Ribosomal protein L23a (RpL23a)	4378006	28.6	10.94	134
2	Ribosomal protein L7a (RpL7a)	22831820	30.7	10.72	115
3	Not ID				
4	Ribosomal protein L6 (RpL6)	24651668	27.7	10.68	68
5	Not ID				
6	Not ID				
7	Ribosomal protein S9 (RpS9)	24661711	22.6	10.61	126
8	Not ID				
9	Histone H3	8070	15.4	11.27	88
10	Not ID				
11	Histone H2A	72151624	13.4	10.72	179
12	Not ID				
13	Histone H4	23171273	11.4	11.35	344

Figure 3.1.9 The fifth set of tandem mass spectrometric (MS/MS) identification of protein bands in the eluent of the affinity pull-down experiment using DBE-coupled beads. On the left panel is the SDS-PAGE gel of the eluent of the affinity pull-down using DBE-coupled beads. Each excised protein bands for MS/MS analysis are labeled with a number and indicated with an arrow. On the right panel is the table showing the summary of MS/MS results. It depicts the number given to each protein band [a], the name of DBE-interactor identified by MS/MS [b], the GenBank molecular biology database identifier (GI no.) [c], the predicted molecular weight, the predicted isoelectric point (pI) [d] and the protein score which was generated by the Mascot software to show the confidence level of the MS/MS result [e]. 'Not ID' denotes the protein was not identified by MS/MS. All the protein scores listed in the table have *p*-values of less than 0.05.



Band no. [a]	DBE-interactor [b]	GI no. [c]	Predicted molecular weight (kDa)	Predicted pI [d]	Protein Score [e]
1	Histone H2A	8072	13.4	10.72	64

Figure 3.1.10 The sixth set of tandem mass spectrometric (MS/MS) identification of protein band in the eluent of the affinity pull-down experiment using DBE-coupled beads. On the left panel is the SDS-PAGE gel of the eluent of the affinity pull-down using DBE-coupled beads. The excised protein band for MS/MS analysis is labeled with a number and indicated with an arrow. On the right panel is the table showing the summary of MS/MS result. It depicts the number given to the protein band [a], the name of DBE-interactor identified by MS/MS [b], the GenBank molecular biology database identifier (GI no.) [c], the predicted molecular weight, the predicted isoelectric point (pI) [d] and the protein score which was generated by the Mascot software to show the confidence level of the MS/MS result [e]. The protein score listed in the table has *p*-value of less than 0.05.

Tandem mass spectrometric results of high confidence and those which showed correct match of protein size on the SDS-PAGE gels were summarized in Figures 3.1.6 to 3.1.11. A total of 31 proteins were identified in the eluents from 6 independent affinity pull-down experiments (Table 3.1). Among them, 15 proteins were consistently identified for at least 3 times (Table 3.1). Ten of them were ribosomal proteins; among them 7 were ribosomal proteins of the large subunit, 2 were ribosomal proteins of the small subunit and 1 is not known for its location in the ribosome (Table 3.1). The other five proteins identified in the eluents were tubulin, LP10071p, actin, DBE and histone H2A (Table 3.1). Tubulin and actin are major components of the cytoskeleton. LP10071p is an uncharacterized protein with a domain of the eukaryotic elongation factor 1 (EF1) alpha subfamily. EF1 is responsible for the GTP-dependent binding of aminoacyl-tRNAs to the ribosomes (Margutti et al., 1999). Histone H2A is one of the core histone proteins, which are components of the nucleosome (Luger et al., 1997).

3.1.2.5 Study of the RNA-dependence of the interactions between DBE and its interactors

Since DBE is a RNA-binding protein (Yiu et al., 2006), the RNA-dependence of the protein interactions of DBE in the affinity pull-down experiments was studied.

Table 3.1 Summary of DBE-interactors identified in the affinity pull-down study

Name of DBE-interactor [a]	No. of times identified by pulled-down assay	Literature cited interaction with Krr1p in yeast [b]	Description [c]
Ribosomal protein L6 (RpL6)	6	-	It is a component of LSU.
Ribosomal protein LP0 (RpLP0)	5	+ (Gavin et al., 2006; Grandi et al., 2002)	It is a component of LSU.
Ribosomal protein L7a (RpL7a)	5	-	It is a component of LSU. The yeast homolog was found in the 90S pre-RNP complex (Kressler et al., 1999).
Ribosomal protein L23a (RpL23a)	5	+ (Gromadka et al., 2004)	It is a component of LSU. The yeast homolog was found in the 90S pre-RNP complex (Kressler et al., 1999).
Actin	4	-	It is a component of cytoskeleton.
Tubulin	4	-	It is a component of cytoskeleton.
Ribosomal protein (Rp)	4	-	Its location in the ribosome is unknown.
Ribosomal protein S9 (RpS9)	4	-	It is a component of SSU. The yeast homolog was found in the 90S pre-RNP complex (Kressler et al., 1999). The human homolog was shown to have similar kinetic profile with DBE homolog HRB2 (Leung et al., 2006).

[a] Name in the bracket is the abbreviation of the DBE-interactor.

[b] '+' denotes the presence of interaction between its homolog in yeast and Krr1p.

'-' denotes the absence of interaction between its homolog in yeast and Krr1p.

[c] 'LSU' denotes the large subunit of ribosome.

'SSU' denotes the small subunit of ribosome.

Table 3.1 (continued) Summary of DBE-interactors identified in the affinity pull-down study

Name of DBE-interactor [a]	No. of times identified by pulled-down assay	Literature cited interaction with Krr1p in yeast [b]	Description [c]
Ribosomal protein S3a (RpS3a)	4	+ (Grandi et al., 2002; Gromadka et al., 2004)	It is a component of SSU.
Ribosomal protein L4 (RpL4)	4	+ (Grandi et al., 2002; Gromadka et al., 2004)	It is a component of LSU. The yeast homolog was found in the 90S pre-RNP complex (Kressler et al., 1999).
Dribble (DBE)	3	-	It is a nucleolar protein involved in ribosome biogenesis.
Histone H2A	3	-	It is a core histone protein.
LP10071p	3	-	It is predicted to belong to elongation factor 1 alpha subfamily.
Ribosomal protein L14 (RpL14)	3	-	It is a component of LSU.
Ribosomal protein L22 (RpL22)	3	-	It is a component of LSU.
Ribosomal protein S3 (RpS3)	1	-	It is a component of SSU.
Histone H3	1	-	It is a core histone protein.
Histone H4	1	-	It is a core histone protein.
Ribosomal protein S8 (RpS8)	1	-	It is a component of SSU.

[a] Name in the bracket is the abbreviation of the DBE-interactor.

[b] '+' denotes the presence of interaction between its homolog in yeast and Krr1p.

'-' denotes the absence of interaction between its homolog in yeast and Krr1p.

[c] 'LSU' denotes the large subunit of ribosome.

'SSU' denotes the small subunit of ribosome.

Table 3.1 (continued) Summary of DBE-interactors identified in the affinity pull-down study

Name of DBE-interactor [a]	No. of times identified by pulled-down assay	Literature cited interaction with Krr1p in yeast [b]	Description [c]
Ribosomal protein S15 (RpS15)	1	-	It is a component of SSU.
Ribosomal protein S16 (RpS16)	1	-	It is a component of SSU.
Ribosomal protein S23 (RpS23)	1	-	It is a component of LSU.
Ribosomal protein L3 (RpL3)	1	-	It is a component of LSU.
Ribosomal protein L12 (RpL12)	1	-	It is a component of LSU.
Ribosomal protein L13 (RpL13)	1	-	It is a component of LSU.
Ribosomal protein L17 (RpL17)	1	-	It is a component of LSU. The yeast homolog was found in the 90S pre-RNP complex (Kressler et al., 1999).
Ribosomal protein L21 (RpL21)	1	-	It is a component of LSU.
Ribosomal protein L26 (RpL26)	1	-	It is a component of LSU.
Ribosomal protein L28 (RpL28)	1	-	It is a component of LSU.
Ribosomal protein L34b (RpL34b)	1	-	It is a component of LSU.
Ribosomal protein L35 (RpL35)	1	-	It is a component of LSU.

[a] Name in the bracket is the abbreviation of the DBE-interactor.

[b] '+' denotes the presence of interaction between its homolog in yeast and Krr1p.
'-' denotes the absence of interaction between its homolog in yeast and Krr1p.

[c] 'LSU' denotes the large subunit of ribosome.
'SSU' denotes the small subunit of ribosome.

Prior to the affinity pull-down, *Drosophila* cell lysates were first treated with RNase A to remove cellular RNAs. Affinity pull-down using cell lysates with and without RNase A treatment were performed in parallel, and their elution patterns were analyzed. In Figure 3.1.11, it showed that most protein bands of the molecular size from 20 kDa to 37 kDa in the eluents were lost or diminished in intensity using protein lysates with RNase A treatment. These proteins were therefore regarded to interact with DBE in an RNA-dependent fashion (Figure 3.3.11). However, intensities of some protein bands in the eluents between affinity pull-down experiments using RNase A-treated and untreated samples remained unchanged (Figure 3.3.12). By means of MS/MS analyses, these proteins were identified to be histone H2A, histone H3 and histone H4 (Figure 3.3.11). Their interactions with DBE were thus regarded as RNA-independent (Figure 3.3.11) indicating that they do not require RNA to interact with DBE.

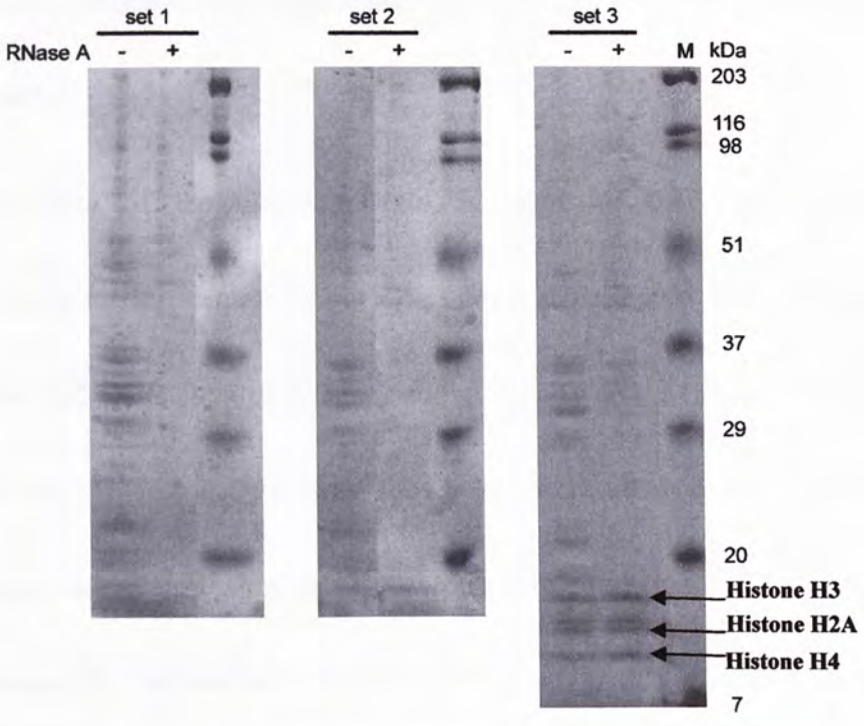


Figure 3.1.11 Study of the RNA-dependence of the interactions in affinity pull-down experiments using DBE-coupled beads. Affinity pull-down were performed using DBE-coupled beads for interacting with protein lysates with '-' and without '+' RNaseA treatment. Eluents in the SDS-PAGE gels from 3 independent sets of affinity pull-down experiments were compared. It showed that most protein bands of the molecular size from 20 kDa to 37 kDa were lost or diminished in intensity using protein lysates with RNase A treatment. In the third set of affinity pull-down experiment, three protein bands which were regarded to interact with DBE in a RNA-independent fashion were identified by MS/MS and found to be histone proteins H2A, H3 and H4.

3.1.3 Discussion

3.1.3.1 Most DBE-interactors pulled down by DBE-coupled beads were shown to be specific

Specificity of the interactors pulled down by DBE-coupled beads was demonstrated by the control affinity pull-down experiments using lysozyme- and heat-denatured DBE-coupled beads (Section 3.1.2.2 and 3.1.2.3). Only 1 protein band pulled down by DBE was found to be resulted from the non-specific electrostatic interaction using the lysozyme control (Section 3.1.2.2). It indicates that non-specific electrostatic interaction resulted in this affinity pull-down was minor. In additions, approximately 20 bands were found to specifically interact with DBE in non-denatured conformation using the heat-denatured DBE control (Section 3.1.2.3). This result further confirmed that most protein bands pulled down by DBE were specific.

Combining the results in Section 3.1.2.2 and 3.1.2.3, three non-specific protein bands were identified including one protein band identified in the control using lysozyme (Figure 3.1.2) and also two protein bands identified in another control using heat-denatured DBE (Figure 3.1.3). According to the molecular size of these

protein bands, the protein band observed in the eluent of the control using lysozyme was of similar molecular size as the upper non-specific protein band in the eluent of the control using heat-denatured DBE (Figure 3.1.2; Figure 3.1.3). Since the molecular size of those three protein bands appeared to be approximately 45 kDa, they might correspond to the bands identified as actin and DBE in the eluents of affinity pull-down using DBE (Figure 3.1.5; Figure 3.1.6; Figure 3.1.8). As actin has a predicted pI value of 5.37 which is lower than the pH of the binding condition of the affinity pull-down, it is possible to interact with basic proteins such as lysozyme and DBE. The presence of DBE in the eluents of the affinity pull-down using DBE-coupled beads is unlikely to be caused by the dimerization of DBE since the low abundance of endogenous DBE would not allow the detection of DBE in the SDS-PAGE gel using Coomassie blue staining. In addition, previous experiment showed that DBE is monomeric using size-exclusion chromatography and sedimentation equilibrium analysis (Yiu et al., 2006). Instead, the presence of DBE in the eluents may be resulted from the release of DBE from resin beads into the eluents. Therefore, actin and DBE identified in the affinity pull-down using DBE-coupled beads were likely to be non-specific.

3.1.3.2 Most of the potential DBE-interactors were found in the nucleolar proteome

Since DBE is localized to the cell nucleolus (Chan et al., 2001), its interactors are expected to be present in the same cellular compartment. Confirming the nucleolar localization of protein interactors of DBE is important since total protein lysates were used in the affinity pull-down. As the protein composition of the nucleolar proteome in *Drosophila* has not been comprehensively studied, the human homologs of DBE protein interactors were search against the Nucleolar Proteome Database (NOPdb) (http://www.lamondlab.com/NoPDB) (Leung et al., 2006). It was found that the human homologs of ribosomal proteins RpS9, RpL4, RpL6, RpL7a, RpL14, RpL22 and RpL23a, histone protein H2A, tubulin and LP10071p in *Drosophila* were present in the human nucleolar proteome. Conversely, three other DBE-interactors ribosomal proteins RpS3a, RpLP0 and one uncharacterized ribosomal protein (Rp) were not found in the human nucleolar proteome which means they may not be localized to the nucleolus or they only present in a very low abundance in the human nucleolus. Nevertheless, those DBE-interactors which were found in the human nucleolus would be of higher possibilities to have interactions with DBE in an *in vivo* environment.

3.1.3.3 Comparison of protein interactions identified in DBE and Krr1p

Though the basic features of ribosome biogenesis are conserved from yeast to higher eukaryotes (reviewed by Venema and Tollervey, 1999), its mechanism is more complex in higher eukaryotes (Lapeyre, 2005) and its pathway shares distinct differences in different organisms (Eichler and Craig, 1994). Likewise, DBE and its homolog in yeast — Krr1p are both involved in rRNA processing, but Krr1p is involved in the processing of 18S rRNA only (Gromadka and Rytka, 2000; Sasaki et al., 2000) whereas DBE is involved in both the 18S and 28S rRNA processing (Chan et al., 2001). Since a known interactor of Krr1p — Faf1p has no clear homolog in flies (Karkusiewicz et al., 2004) and the mutant of *faf1* has similar defects of rRNA processing as that of the *krr1* mutant (Karkusiewicz et al., 2004), it is possible that the interaction between Faf1p and Krr1p is important for performing rRNA processing in yeast. Therefore, it is expected that DBE might interact with other proteins which are important for its rRNA processing. The dissimilarities between the interaction profiles of DBE and Krr1p might help to explain the difference in rRNA processing defects between *dbe* and *krr1* mutants.

Unlike the present study which used purified DBE as bait for affinity pull-down, tandem affinity purification (TAP) which involves the expression of tagged Krr1p in

yeast cells was used to identify interactors of Krr1p (Gavin et al., 2006; Grandi et al., 2002; Gromadka et al., 2004). Though the strategies used in the interaction studies of Krr1p and DBE were different, common protein interactors were found between the interaction profiles of DBE and Krr1p. By comparing the DBE-interactors identified in the present study with the Krr1p-interactors reported in the BioGRID database (http://www.thebiogrid.org) (Stark et al., 2006), 4 out of 13 DBE-interactors have also been identified to interact with Krr1p which include the yeast homologs of RpS3a, RpLP0, RpL4 and RpL23a in *Drosophila* (Table 3.1). Apart from the study of physical interaction of Krr1p, the homolog of a DBE-interactor — RpS9 was also found to genetically interact with *krr1* mutant (Gromadka et al., 2004).

The yeast homologs of nine DBE-interactors identified in this study were not identified to have interactions with Krr1p (Table 3.1). Among them, 6 of these protein are ribosomal proteins, and the others include tubulin, an uncharacterized protein with a domain of the eukaryotic elongation factor 1 (EF1) alpha subfamily and histone H2A (Table 3.1). The importance of the interactions between these 6 proteins and DBE would need further investigation. In particular, the interactions between histone proteins and Krr1p have not been reported in yeast. Histone proteins are known to be transcriptional regulators which govern the gene activation

or repression (Berger, 2002). Whether the interaction between histone proteins and DBE are important for regulating gene expressions is yet to be investigated. To further confirm the interactions between histone proteins and DBE, co-immunoprecipitation and co-sedimentation study using sucrose gradient sedimentation were performed (Section 3.4).

3.1.3.4 The implications of DBE-interacting ribosomal proteins on the role of DBE in ribosome biogenesis

It seems that the DBE-interactors identified in this study (Table 3.1) have no obvious and direct role in rRNA processing since they do not carry any enzymatic domains that are responsible for rRNA cleavages or modifications. However, some ribosomal proteins were reported to be involved in rRNA processing as the depletion of a number of ribosomal proteins was found to cause distinct rRNA processing defects (Ferreira-Cerca et al., 2005; Leger-Silvestre et al., 2004). Evidence indicates that DBE is involved in the early phase of ribosome biogenesis as depletion of DBE was shown to affect the early pre-rRNA cleavage in rRNA processing (Chan et al., 2001). Therefore, it is important to find out whether the subset of DBE-associating ribosomal proteins (Table 3.1) is involved in the early phase of ribosome biogenesis.

It is known that some ribosomal proteins are assembled into the earliest detectable 90S pre-rRNP complex, which are also known as early-associating ribosomal proteins (reviewed by Kressler et al., 1999). Based on Kressler et al. (1999), the yeast homologs of 5 DBE-interacting ribosomal proteins identified in the present study were found to be present in the 90S pre-RNP complex (Table 3.1). They include the RpS9, RpL4, RpL7A and RpL23a (Table 3.1). These findings showed some of the DBE-interacting ribosomal proteins are involved in the early phase of ribosome biogenesis. Since DBE was found to be involved in the early maturation step of the rRNA processing (Chan et al., 2001), it is possible that the association of DBE with these early-associating ribosomal proteins are important for the rRNA processing. The exact function of the association of DBE and these ribosomal proteins is yet to be investigated. Moreover, DBE could be involved in ribosome assembly which helps ribosomal proteins to assemble into pre-rRNP complex. If this hypothesis is true, it might indicate that DBE associates with pre-rRNP complex. As an initial step to test this hypothesis, sucrose gradient sedimentation was performed to study the sedimentation behavior of DBE to determine whether DBE associates with a macromolecular complex (Section 3.2). It is expected that proteins which associate with a macromolecular complex would

sediment at sucrose fractions of high sucrose density.

Accordingly, a study was conducted to investigate the dynamics of the human nucleolar proteome in response to the inhibition of rDNA transcription by actinomycin D (Andersen et al., 2005). The kinetic profiles of the nucleolar localizations of proteins and those which share similar kinetic behaviors were reported in the Nucleolar Proteome Database (NOPdb) (Leung et al., 2006). To gain clues for the association between DBE and its interactors, the kinetic profile of the DBE homolog in human — HRB2 after the inhibition of rDNA transcription was retrieved from NOPdb for analysis (Leung et al., 2006). It is suggested that proteins which leave the nucleolus in similar kinetics after transcriptional inhibition would likely be involved in similar steps of ribosome biogenesis (Andersen et al., 2005). It was found that RpS9 and HRB2 share similar kinetic profiles and hence it suggests that DBE and RpS9 may participate in similar steps of ribosome biogenesis (Leung et al., 2006). Since the physical interaction between RpS9 and DBE has not been reported in other studies, it is worthwhile to confirm the interaction between them. GST pull-down assay was therefore performed to study the interaction between RpS9 and DBE (Section 3.3).

3.2 Study of the sedimentation behavior of DBE by sucrose gradient sedimentation

3.2.1 Introduction

DBE was consistently found to associate with 10 ribosomal proteins in the affinity pull-down experiments (Table 3.1) and 4 of the DBE-interacting ribosomal proteins including RpS9, RpL4, RpL7a and RpL23a were found to be involved in the early phase of ribosome biogenesis (reviewed by Kressler et al., 1999). Moreover, DBE is involved in the early phase of rRNA processing (Chan et al., 2001). Since rRNA processing was found to proceed with the addition of ribosomal proteins and other processing factors such as rRNA modifying enzymes onto pre-rRNAs to give large pre-rRNP complexes (reviewed by Kressler et al., 1999), it is hypothesized that DBE may associate with some ribosomal proteins and exists in a pre-rRNP complex. As the first step to test this hypothesis, sucrose gradient sedimentation was employed to determine whether DBE exists in a large complex by studying the sedimentation behavior of DBE. In sucrose gradient sedimentation, the density of a protein complex would determine its sedimentation behavior in the sucrose gradient. A macromolecular complex of high density would sediment at denser sucrose fractions while free protein molecules would remain at the top region of the gradient. Hence,

it is expected that DBE would sediment at denser sucrose fractions if it associates with a macromolecular complex in cells.

3.2.2 Results

3.2.2.1 The sedimentation behaviors of endogenous DBE and overexpressed FLAG-DBE

Drosophila fly head lysates obtained from wild-type flies were layered onto the top of a continuous sucrose gradient for sedimentation. After ultracentrifugation, sucrose fractions were recovered for SDS-PAGE and Western blot analyses. Anti-DBE antibody was used to detect DBE protein, and found that DBE sedimented at the 5^{th} to 7^{th} sucrose fractions which corresponded to approximately 35 - 40% of sucrose (Figure 3.2A). However, the DBE protein bands detected were faint which might be due to the low abundance of endogenous DBE protein in *Drosophila* (Chan et al., 2001). Therefore, fly head lysates with overexpressed FLAG-DBE were used for sucrose gradient sedimentation. The sedimentation behaviors of DBE and FLAG-DBE were found to be similar. It was observed that the protein band intensities of FLAG-DBE in the 5^{th} to 7^{th} sucrose fractions were much enhanced (Figure 3.2B). Since DBE would not have a high density to sediment at fractions of

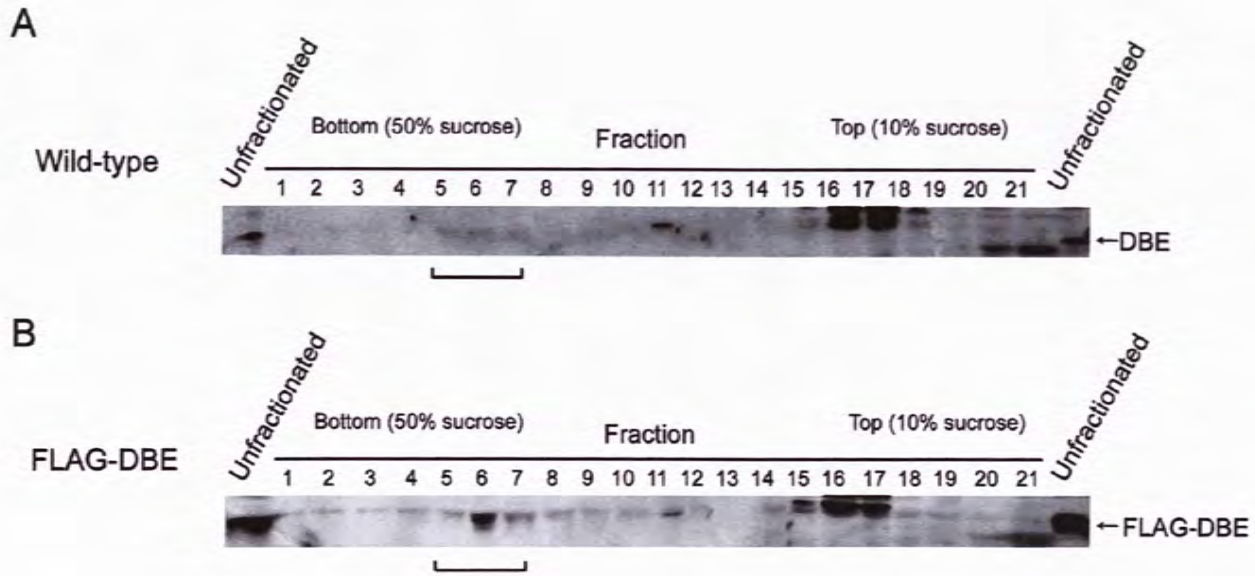


Figure 3.2 Sedimentation behaviors of DBE and FLAG-DBE. Sucrose gradient sedimentation study of **(A)** wild-type fly head lysates and **(B)** fly head lysates with overexpressed FLAG-DBE. Each sucrose fraction (~0.5 ml) was collected from bottom to top of a continuous 10% to 50% sucrose gradient after ultracentrifugation. The fraction numbers were counted from bottom to top (i.e., fraction 1 corresponds to 50% sucrose, while fraction 21 corresponds to 10% sucrose). A total of 21 fractions were recovered for SDS-PAGE and Western blot analyses. Membranes were probed with anti-DBE antibody. Protein bands that correspond to DBE and FLAG-DBE are indicated with arrows. The 5th to 7th sucrose factions which contained the endogenous DBE **(A)** and their corresponding fractions in **(B)** are indicated with brackets.

high sucrose density if it exists in a monomeric form, DBE was likely to exist in a form that associates with a macromolecular complex.

3.2.3 Discussion

3.2.3.1 The sedimentation behaviors of endogenous DBE and overexpressed FLAG-DBE were similar

In the comparison between the sedimentation behaviors of endogenous DBE and overexpressed FLAG-DBE, it was shown that substantial amount of FLAG-DBE was still found in 5^{th} to 7^{th} sucrose fractions which was similar to that of the endogenous DBE (Figure 3.2). This result demonstrated that the FLAG-tag fused with DBE as well as the FLAG-DBE overexpression would not have major effects on the sedimentation behavior of DBE. However, it should be noted that some faint bands of FLAG-DBE were observed in the 1^{st} to 4^{th} and 8^{th} to 10^{th} sucrose fractions (Figure 3.2B), those faint bands were not observed in the sucrose gradient using wild-type fly head lysates (Figure 3.2A). These faint bands might represent that overexpressed FLAG-DBE associates with other macromolecular complexes of other densities. On the other hand, these macromolecular complexes might have a weak association with endogenous DBE. Since the major bands of the endogenous DBE

in the 5th to 7th sucrose fractions were faint (Figure 3.2A), the protein level of the endogenous DBE in the 1st to 4th and 8th to 10th sucrose fractions might be too low to be detected when wild-type fly head lysates were used.

3.2.3.2 DBE associates with a macromolecular complex

In this study, DBE was observed to sediment at the denser sucrose fractions of the sucrose gradient which had approximately 35 – 40% of sucrose. The sedimentation behavior of DBE suggested that DBE is associated with a macromolecular complex which has a high density to sediment to the fractions containing high density of sucrose. It is because if DBE exists as a free monomeric protein, it would be found at the top region of the sucrose gradient. To find out the identity of the macromolecular complex which associates with DBE, a ribosome profile can be obtained by taking the OD_{260} readings of the sucrose fractions (Harnpicharnchai et al., 2001). The ribosome profile will give several peaks representing the pre-rRNP complexes and ribosomal subunits (Harnpicharnchai et al., 2001). Hence, the identity of the DBE-containing complex can be predicted by referring the fractions containing the DBE-containing complex to the ribosome profile. Alternatively, the 5th to 7th sucrose fractions of the sucrose gradient containing overexpressed FLAG-DBE could be used to pull down the

macromolecular complex. The protein components of the macromolecular complex can then be analyzed by MS/MS to see if it contains the subset of ribosomal proteins which interact with DBE and other DBE-interactors which were identified in the affinity pull-down (Table 3.1). Since DBE is a KH-domain containing protein which was demonstrated to associate with RNA *in vitro* (Yiu et al., 2006), it is likely that the DBE-containing complex also contains RNA. More importantly, the identities of pre-rRNP complexes are often distinguished by the type of pre-rRNA they contain (Grandi et al., 2002; Harnpicharnchai et al., 2001). The RNA components of the DBE-containing complexes can be identified by primer extension and Northern Blot analyses using RNA which co-immunoprecipitated with the FLAG-DBE-containing complex. The identity of the DBE-containing complex would reveal whether DBE exists in a pre-rRNP complex. Moreover, the importance of the interaction between DBE and ribosomal proteins can then be investigated to determine whether DBE would be involved in ribosome assembly which helps ribosomal proteins to assemble into pre-rRNP complexes.

3.3 Study of the interaction between DBE and RpS9 by GST pull-down assay

3.3.1 Introduction

In the affinity pull-down experiment, RpS9 was consistently found to interact with DBE (Table 3.1). Further analyses suggest that DBE and RpS9 have a close relationship. First, the human homologs of DBE and RpS9 were observed to display similar kinetics of nucleolar localization after the inhibition of rDNA transcription (Andersen et al., 2005). This finding suggests that DBE and RpS9 may participate in ribosome biogenesis at similar stage. Second, the yeast homolog of *rps9* was shown to genetically interact with the homolog of *dbe* in yeast — *krr1* (Gromadka et al., 2004). The overexpression of *rps9* in yeast was shown to rescue the growth defect of a cold sensitive *krr1* mutant which has a sequence deletion in the predicted nuclear localization signal (Gromadka et al., 2004). Since the physical interaction between the protein family of DBE and RpS9 has not been reported so far, confirming their direct physical interaction is essential before further characterizing the functional importance of their interaction. In this study, GST pull-down assay was employed to confirm the interaction between DBE and RpS9 identified in the affinity pull-down (Table 3.1).

3.3.2 Results

3.3.2.1 The constructs for GST and GST-RpS9 expressions

In the GST pull-down assay, the constructs pGEX-6p-1 and pGEX-6p-1-*rps9* were used to express GST and GST-RpS9 respectively. The construct pGEX-6p-1-*rps9* has an open-reading frame of *rps9* subcloned into the expression vector pGEX-6p-1. The open-reading frame of the *rps9* originates from the *Drosophila rps9* cDNA which had been cloned into pRSETA-GST-EK and its sequence had been confirmed by DNA sequencing.

3.3.2.2 Purified DBE protein was pulled down by GST-RpS9

In the GST pull-down assay, GST-RpS9 was used as a bait to bind purified DBE protein. This assay was performed in a reciprocal format to the affinity pull-down in which purified DBE protein was used as a bait (Section 3.1). GST was used as a negative control to demonstrate the interaction between DBE and GST-RpS9 is specific. Bacterial lysates with the overexpressions of GST and GST-RpS9 were loaded to Glutathione Sepharose 4 Fast Flow beads respectively before binding with purified DBE protein. The beads loaded with GST or GST-RpS9 were incubated

with purified DBE protein and then washed extensively to get rid of any unbound DBE. The result of GST pull-down assay showed that GST pulled down minute amount of DBE (Figure 3.3), whereas GST-RpS9 pulled down significantly more DBE (Figure 3.3). Since the bands for GST and GST-RpS9 in the eluents showed similar intensities indicating that the amount of the bait used in each GST pull-down assay was comparable (Figure 3.3), DBE was shown to interact with RpS9 directly.

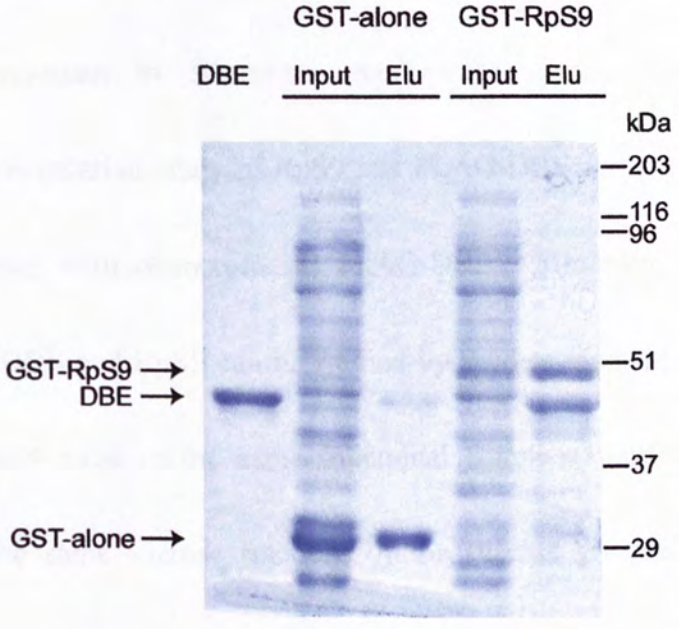


Figure 3.3 SDS-PAGE analysis of the GST pull-down assay. Bacterial lysates with the GST and GST-RpS9 expressed were loaded to Glutathione Sepharose 4 Fast Flow beads respectively before binding with purified DBE. The beads loaded with GST or GST-RpS9 were incubated with purified DBE. The eluents (Elu) of GST pull-down assay experiments showed that GST pulled down minute amount of DBE, whereas GST-RpS9 pulled down significantly more DBE. The protein bands corresponding to DBE (~40 kDa), GST (~26 kDa) and GST-RpS9 (~50 kDa) are indicated with arrows.

3.3.3. Discussion

3.3.3.1 Further investigations on the interaction between DBE and RpS9

After confirming the physical interaction between DBE and RpS9 *in vitro*, it would be important to determine whether their interaction exists *in vivo*. Co-immunoprecipitation study of RpS9 and FLAG-DBE could be performed using fly head lysates with overexpressed FLAG-DBE. Moreover, the sedimentation behaviors of DBE and RpS9 can be studied by sucrose gradient sedimentation. If DBE and RpS9 exist in the same functional complex, they would co-sediment together at the same sucrose fractions of the sucrose gradient. Confirming the physical interaction between DBE and RpS9 *in vivo* might explain their similar kinetics of nucleolar localization after the inhibition of rDNA transcription (Leung et al., 2003), because they might associate with each other and delocalize together from the nucleolus.

3.3.3.2 The implications of interaction between DBE and RpS9 on ribosome biogenesis

Previous study showed that DBE is involved in the early cleavage step in rRNA processing (Chan et al., 2001). It is known that some ribosomal proteins implicate

also in rRNA processing viewing that the depletion of some ribosomal proteins causes distinct rRNA processing defects (Ferreira-Cerca et al., 2005; Leger-Silvestre et al., 2004). Since the yeast homolog of the *Drosophila* RpS9 is an early-assembling ribosomal protein and it is present in the earliest detectable 90S pre-RNP complex during ribosome assembly (reviewed by Kressler et al., 1999), RpS9 may also affect the early cleavage step in rRNA processing because ribosome assembly and rRNA processing are two interdependent processes (reviewed by Granneman and Baserga, 2005; Tschochner and Hurt, 2003). Hence, the interaction between DBE and RpS9 identified in this study could be important for the involvement of DBE in the early cleavage step in rRNA processing.

On the other hand, the association between DBE and RpS9 might indicate a role of DBE in assisting the assembly of RpS9 into pre-rRNP complexes. At present, the order and mechanism of ribosomal assembly remain obscure (Fromont-Racine et al., 2003). It is suggested that ribosomal proteins need to interact with rRNA in a sequence-specific manner for their correct assembly into pre-rRNP complexes (Draper and Reynaldo, 1999). RpS9 has a putative S4 RNA-binding domain (Aravind and Koonin, 1999) and it is predicted to bind rRNA like many other RNA-binding ribosomal proteins (Draper and Reynaldo, 1999). However, some

ribosomal proteins have been shown to require other components to associate with rRNA (Nomura, 1973). The interaction between DBE and RpS9 may be required for the specific binding of RpS9 to rRNA or it may also implicate in some regulatory processes for controlling the assembly of RpS9 into pre-rRNP complexes.

To understand if the interaction between RpS9 and DBE is involved in rRNA processing and ribosome assembly, the genes *dbe* and *rps9* can be knocked-down respectively in *Drosophila* cultured cells to see if each of them would give similar defects in rRNA processing and ribosome assembly. To detect defects in rRNA processing, total RNA can be extracted from cells for Northern Blot analysis to detect the presence of aberrant pre-rRNAs or rRNAs (Chan et al., 2001). For the detection of defects in ribosome assembly, protein lysates can be extracted from cells to perform sucrose gradient sedimentation. By taking the OD_{260} readings of the sucrose fractions, it will give a ribosome profile showing the abundance of pre-RNP complexes and mature ribosomal subunits (Harnpicharnchai et al., 2001). Decreased levels of pre-RNP complexes or mature ribosomal subunits in the ribosome profile would indicate defects in ribosome assembly. If DBE is required for the assembly of RpS9 into pre-rRNP complexes, *dbe* null mutants would give ribosome assembly defects. Furthermore, sucrose gradient fractions can be

subjected to Western blot analysis to study the sedimentation behavior of RpS9 in *dbe* null mutants. It is expected that the sedimentation behavior of RpS9 would be altered in the absence of DBE if RpS9 cannot be assembled into pre-rRNP complexes without the assistance of DBE.

3.3.3.3 The RNA dependence of the interaction between RpS9 and DBE

Although it was shown that DBE-associating ribosomal proteins including RpS9 interacted with DBE in an RNA-dependent manner in the affinity pull-down experiment (Figure 3.1.11), RpS9 was shown to bind DBE in the GST pull-down assay without the supplement of cellular RNA to the binding reaction (Figure 3.3). These results can be explained by the fact that more RpS9 protein was used in the GST pull-down assay than the affinity pull-down since RpS9 in the protein input of the GST pull-down assay was overexpressed in *E. coli*. Therefore, it is possible that the lack of interaction between RpS9 and DBE in the absence of RNA in the affinity pull-down might have only been due to the weakening of the interaction between RpS9 and DBE (Figure 3.1.11). When RpS9 was present in large amount in the GST pull-down assay, DBE was pulled down even in the absence of RNA (Figure 3.3). To test whether RNA would increase the binding affinity of RpS9 towards DBE in the GST pull-down assay, cellular RNA can be added to the binding reaction

to observe whether more DBE are pulled down by GST-RpS9.

3.4 Study of the association between DBE and histone proteins by co-immunoprecipitation and sucrose gradient sedimentation

3.4.1 Introduction

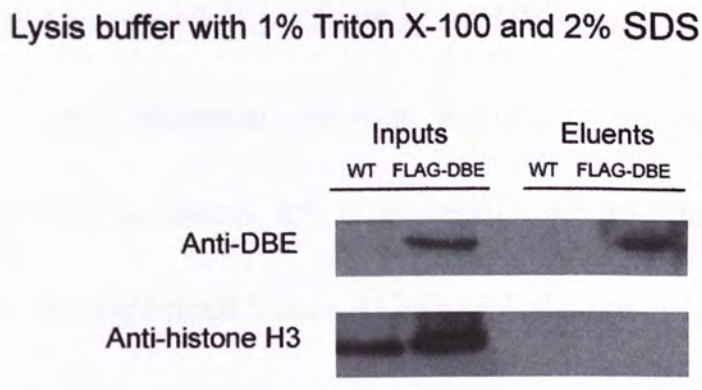
From the affinity pull-down experiments, histone proteins H2A, H3 and H4 were found to interact with DBE (Table 3.1) and they all belong to the family of core histone proteins, which interact with each other in the nucleosome (Luger et al., 1997) and function as transcriptional regulators in cells (Berger, 2002). Since their interactions with the protein family of DBE have not been reported so far (Section 3.1.3.3), histone interactions with DBE might implicate unknown roles of DBE besides rRNA processing. Thus, it is crucial to confirm the interactions between histone proteins and DBE found in the affinity pull-down experiments (Table 3.1). Since only antibody against *Drosophila* histone H3 was available commercially, histone H3 was chosen for studying its association with DBE by means of co-immunoprecipitation and sucrose gradient sedimentation. It is hypothesized that if histone H3 interacts with DBE *in vivo*, histone H3 could be co-immunoprecipitated with DBE. Similarly, if histone H3 presents in the same complex of which DBE associates, it would co-sediment with DBE at the same sucrose fractions in the sucrose gradient sedimentation.

3.4.2 Results

3.4.2.1 Histone H3 was not co-immunoprecipitated with FLAG-DBE

Fly head lysates with overexpressed FLAG-DBE were used for co-immunoprecipitation, and wild-type fly head lysates without overexpressed FLAG-DBE were used as negative control. The fly lysates were allowed to bind with anti-FLAG M2 beads and the unbound proteins were then washed away. Any proteins that bound to the anti-FLAG M2 beads were subsequently eluted by heating the beads in SDS sample buffer, and the eluents were then subjected to SDS-PAGE and Western blot analyses. Anti-DBE antibody was used to detect FLAG-DBE in the eluents to confirm FLAG-DBE was specifically pulled down in the co-immunoprecipitation experiment (Figures 3.4.1). However, no histone H3 was found in the eluents when lysis buffer containing 1% Triton X-100 and 2% SDS was used (Figures 3.4.1A). It was noted that histone H3 was detected in the protein inputs for the co-immunoprecipitation (Figures 3.4.1A) and thus the absence of histone H3 in the eluents would not be due to the low abundance of histone H3 in the cell lysates. Since the binding condition of the co-immunoprecipitation might not be optimal, a "weaker" lysis buffer (1% NP-40 and 1% SDS) was therefore used. However, histone H3 protein was detected in both the eluents of the FLAG-DBE

A.



B.

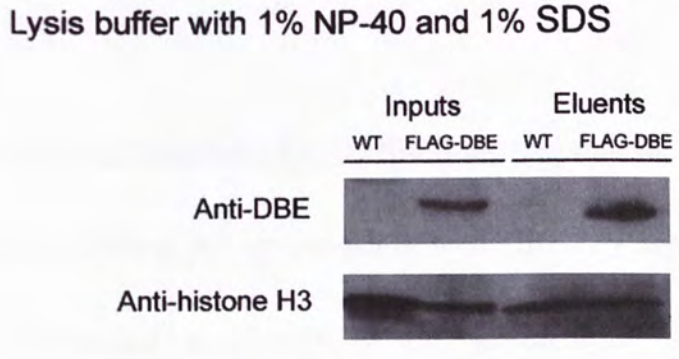


Figure 3.4.1 Co-immunoprecipitation of FLAG-DBE and histone H3. Co-immunoprecipitations were performed using fly head lysates with overexpressed FLAG-DBE and the negative controls were performed using wild-type fly head lysates (WT). The binding and washing steps in the co-immunoprecipitaions were carried out in lysis buffer containing 1% Triton X-100 and 2% SDS **(A)** and lysis buffer containing 1% NP-40 and 1% SDS **(B)**. The inputs and eluents of the experiments were subjected to SDS-PAGE and Western blot analyses. Membranes were probed with anti-DBE and anti-histone H3 antibodies respectively. Histone H3 was not found to be co-immunoprecipitated with FLAG-DBE.

lysates as well as the negative control (Figures 3.4.1B). Since FLAG-DBE was not present in the eluent of the negative control (Figures 3.4.1B), this indicates that histone H3 interacted non-specifically with anti-FLAG M2 beads when the "weaker" lysis buffer was used. Moreover, the band intensity of histone H3 in the FLAG-DBE lysate was the same as that in the negative control (Figures 3.4.1B). Thus, there should be no additional histone H3 being pulled down by FLAG-DBE in the co-immunoprecipitation using lysates with overexpressed FLAG-DBE. To conclude, histone H3 was not found to be co-immunoprecipitated with FLAG-DBE.

3.4.2.2 Histone H3 did not co-sediment with DBE in sucrose gradient sedimentation

To test whether histone H3 co-sediments with DBE, fly head lysates with FLAG-DBE overexpression were prepared and subjected to sucrose gradient sedimentation. As demonstrated in Section 3.2, sedimentation behaviors of endogenous DBE and overexpressed FLAG-DBE were similar except that the signal was much enhanced when lysates with overexpressed FLAG-DBE were used (Figure 3.2). After ultracentrifugation, sucrose fractions were collected for SDS-PAGE and Western blot analyses. Both anti-DBE and anti-histone H3 antibodies were used to detect DBE and histone H3 proteins respectively. DBE was mainly detected from the 3rd to 6th sucrose fractions, while histone H3 was mainly detected from the 1st to

3rd and the 9th to 14th sucrose fractions (Figure 3.4.2). Though a minority of histone H3 was detected in the 3rd to 6th sucrose fractions (Figure 3.4.2), the sedimentation profile of DBE was distinct from that of histone H3. It indicates that a majority of histone H3 did not co-sediment with FLAG-DBE.

3.4.3 Discussion

3.4.3.1 Association between DBE and histone H3 could not be found

Based on the co-immunoprecipitation and the sedimentation studies, association between DBE and histone H3 could not be detected since histone H3 was not co-immunoprecipitated with FLAG-DBE (Figure 3.4.1) and histone H3 did not co-sediment with FLAG-DBE (Figure 3.4.2). It is known that histone proteins can have different patterns of post-translational modifications such as acetylation, methylation and ubiquitination, which would affect their interactions with proteins (Grunstein, 1997; Strahl and Allis, 2000). Hence, histone H3 might have a different state of post-translational modifications and was not co-immunoprecipitated with FLAG-DBE. In the sedimentation study, histone H3 was also found in the sucrose fractions containing FLAG-DBE though a majority of histone H3 was found in other

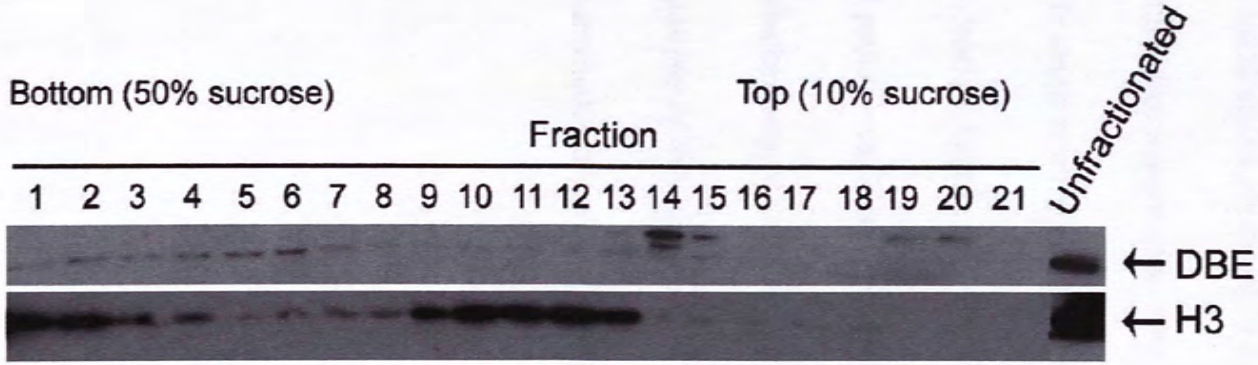


Figure 3.4.2 Sedimentation behaviors of FLAG-DBE and histone H3. Sucrose gradient sedimentation of fly head lysates with overexpressed FLAG-DBE was performed. Each sucrose fraction (~0.5 ml) was collected from bottom to top of a continuous 10% to 50% sucrose gradient after ultracentrifugation. The fraction numbers were counted from bottom to top (i.e., fraction 1 corresponds to 50% sucrose, while fraction 21 corresponds to 10% sucrose). A total of 21 fractions were recovered for SDS-PAGE and Western blot analyses. Membrane was probed with anti-DBE and anti-histone H3 antibodies respectively. Protein bands that correspond to FLAG-DBE and histone H3 are indicated with arrows. The sedimentation behavior of FLAG-DBE was distinct from that of histone H3.

sucrose fractions (Figure 3.4.2). Thus, it cannot exclude the possibility that histone H3 associates with DBE-containing complex in cells.

3.4.3.2 Further investigation on the association between DBE and histone proteins

Though antibodies against other *Drosophila* histone proteins like histone H2A are not available commercially, epitope tags can be introduced to histone proteins for expressions in *Drosophila* cells for co-immunoprecipitation study. Cell lysates with tagged histone proteins can also be subjected to sucrose gradient sedimentation for investigating whether they co-sediment with DBE. These experiments would allow further investigations of the interaction between DBE and other histone proteins *in vivo* and find out whether they co-exist in the DBE-containing complex.

4. GENERAL DISCUSSION

Ribosome biogenesis takes place in the nucleolus and involves two coupled and co-regulated processes — rRNA processing and ribosome assembly (reviewed by Granneman and Baserga, 2005). Ribosomal RNA processing proceeds with the addition of ribosomal proteins onto pre-rRNAs to give pre-rRNP complexes (reviewed by Kressler et al., 1999). Apart from ribosomal proteins and pre-rRNAs, many non-ribosomal proteins are also involved in various steps of ribosome biogenesis (reviewed by Fromont-Racine et al., 2003; Takahashi et al., 2003). The precise roles of these non-ribosomal proteins in ribosome biogenesis remain poorly characterized (Coute et al., 2006). Understanding the physical interaction network among non-ribosomal proteins, ribosomal proteins and pre-rRNAs would provide important insights into the underlying mechanisms of ribosome biogenesis.

Dribble is a non-ribosomal nucleolar protein which is involved in an early cleavage step in rRNA processing (Chan et al., 2001). Moreover, DBE carries a RNA-binding domain and was shown to bind RNAs (Yiu et al., 2006). However, DBE lacks any known enzymatic domain in its sequence to account for its role in rRNA processing. Hence, it is speculated that DBE recruits processing factors onto

the correct sites along rRNAs for rRNA processing. In an attempt to find out these processing factors, affinity pull-down experiment was employed to capture protein interactors of DBE (Section 3.1).

In the affinity pull-down experiments, approximately 22 proteins were pulled down by DBE (Figure 3.1.2; Figure 3.1.3). Control experiments using lysozyme and heat-denatured DBE for affinity pull-down demonstrated respectively that approximately 20 proteins were specifically isolated and they interacted with DBE only in its non-denatured conformation (Figure 3.1.2; Figure 3.1.3). These 20 protein candidates are designated as potential DBE-interactors. In the MS/MS analysis, the identities of 13 of these DBE-interactors were uncovered (Table 3.1). Ten of the identified DBE-interactors have their human homologs identified in the human nucleolar proteome (Leung et al., 2006), including 7 ribosomal proteins RpS9, RpL4, RpL6, RpL7a, RpL14, RpL22 and RpL23a, and three other proteins including histone H2A, tubulin and LP10071p which is an uncharacterized protein with a domain of the eukaryotic elongation factor 1 (EF1) alpha subfamily (Section 3.1.2.4). These proteins would be strong candidates to interact with DBE in an *in vivo* environment since DBE is also a nucleolar-localized protein. Meanwhile, DBE is involved in the early cleavage step in rRNA processing (Chan et al., 2001), while 4

of the identified ribosomal proteins including RpS9, RpL4, RpL7a and RpL23a (Section 3.1.3.4) have their yeast homologs found in 90S pre-rRNP complex which implicates in the early phase of ribosome biogenesis (reviewed by Kressler et al., 1999). One of these ribosomal proteins — RpS9 was further demonstrated to interact directly with DBE in the GST pull-down assay (Figure 3.3). The interaction between DBE and RpS9 or other ribosomal proteins identified could be important for the early cleavage step in rRNA processing as a number of ribosomal proteins like RpL32 had been shown previously to implicate in rRNA processing (Ferreira-Cerca et al., 2005; Leger-Silvestre et al., 2004; Vilardell and Warner, 1997). Moreover, the association of DBE with ribosomal proteins might indicate a role of DBE in assisting the assembly of ribosomal proteins into pre-rRNP complexes.

The affinity pull-down employed in the present study to find DBE-interactors is different from the tandem affinity purification (TAP) used for finding protein interactors of the DBE homolog in yeast — Krr1p (Gavin et al., 2006; Grandi et al., 2002; Gromadka et al., 2004). Despite different strategies were used, comparison between the protein interaction profiles of DBE and Krr1p revealed four common proteins including ribosomal proteins RpS3a, RpLP0, RpL4 and RpL23a (Table 3.1) which are conserved protein interactions in *Drosophila* and yeast. More

importantly, the present approach made use of purified DBE protein which interacts with proteins present in cell lysates. This is distinct from traditional pull-down experiment which uses antibodies for immunoprecipitation. The present approach used in this study would allow new interactors to be identified through different settings of experiment. Some DBE-interactors have not yet been found in Krr1p such as histone proteins (Table 3.1) and they might represent protein interactions that are not present in unicellular eukaryotes or novel interactors being found for the protein family of DBE. Among them, histone proteins were selected for further investigation. Since histone proteins are transcriptional regulators in cells (Berger, 2002), it might implicate some unknown functions of DBE such as the regulation of gene expression. However, histone H3 was neither co-immunoprecipitated with DBE (Figure 3.4.1) nor was it found to co-sediment with DBE in the sucrose gradient sedimentation experiment (Figure 3.4.2). Further studies would be required to elucidate the association between DBE and other histone proteins like histone H2A.

In the sucrose gradient sedimentation experiments, DBE was found to sediment at sucrose fractions of high sucrose density (Figure 3.2). This observation suggests that DBE exists in a macromolecular complex in cells (Figure 3.2). Since DBE is

involved in rRNA processing (Chan et al., 2001) and DBE was found to interact with a number of ribosomal proteins, it is postulated that DBE exist in a ribosomal protein-rich pre-rRNP complex where the rRNA processing takes place (Kressler et al., 1999). Moreover, this favors the hypothesis that the 13 DBE-interactors found in this study might exist in the same complex or different complexes containing DBE rather than having independent interaction with DBE individually. Thus, it is essential to reveal the identity and the composition of the DBE-containing complex to verify these postulations.

Initially, it is hypothesized that DBE might associate with other enzymatic proteins required for rRNA processing, and such protein interactions would explain the aberrant rRNA intermediates observed in *dbe* mutants (Chan et al., 2001). Although the 13 DBE-interactors found in the affinity pull-down have no obvious and direct role in RNA metabolism and most of them were identified to be ribosomal proteins, It is known that some ribosomal proteins are involved in distinct cleavage steps in rRNA processing (Ferreira-Cerca et al., 2005; Leger-Silvestre et al., 2004). In addition, the correct assembly of pre-rRNP complexes was found to be important for rRNA processing to proceed correctly (Harnpicharnchai et al., 2001). The direct interaction between one of the DBE-interacting ribosomal proteins — RpS9

suggests that the RNA-binding DBE might be involved in ribosome assembly which helps RpS9 to associate with rRNA for its assembly into pre-rRNP complex. Thus, it is speculated that the rRNA cleavage defects in *dbe* mutants might be caused by the defects in ribosome assembly when there is no DBE to guide its interacting ribosomal proteins to rRNA to give the correct formation of pre-rRNP complex.

In summary, DBE was found to exist in a macromolecular complex in cells and interact with a number of ribosomal proteins. In addition, DBE was shown to interact directly with ribosomal protein RpS9 which would give crucial hints on the molecular role of DBE in ribosome biogenesis. To further understand the relationship between the protein interactions of DBE and the DBE-containing complex identified in sucrose gradient sedimentation, constituents of the DBE-containing complex could be studied in the future to see if it contains the DBE-interactors identified in the affinity pull-down.

5. CONCLUSION

This project aims to investigate the role of DBE through identifying its protein interactors. Twenty proteins were found to interact specifically with DBE by affinity pull-down experiment. Tandem mass spectrometric analysis revealed the identities of 13 DBE-interactors. Analyses showed that four of the DBE-interactors which include ribosomal proteins RpS9, RpL4, RpL7A and RpL23a have their homologs identified in the human nucleolar proteome and are involved in the early phase of ribosome biogenesis similar to DBE (Chan et al., 2001; Kressler et al., 1999; Leung et al., 2006). Hence, these four proteins would be strong candidates to interact with DBE in an *in vivo* environment and to be involved in similar processes with DBE in ribosome biogenesis. The interaction between DBE and ribosomal proteins suggests a possible role of DBE in assisting the assembly of ribosomal proteins into pre-rRNP complexes. Consistent with this, direct interaction between DBE and ribosomal protein RpS9 was confirmed in this study using GST pull-down assay. In addition, sucrose gradient sedimentation analysis showed that DBE sedimented at denser sucrose fractions which suggested DBE exists in a macromolecular complex in cells.

In summary, DBE was found to interact with a number of ribosomal proteins and exist in a macromolecular complex. Further investigations on the importance of DBE-ribosomal protein interactions in ribosome biogenesis would better define the molecular role of DBE. Moreover, identification of the DBE-containing complex would allow us to determine whether DBE associates with a pre-rRNP complex.

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